UTILITY PATENT APPLICATION

FOR

COMPOSITIONS AND METHODS FOR INDUCTION OF PROTEINS INVOLVED IN XENOBIOTIC METABOLISM

by

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COMPOSITIONS AND METHODS FOR INDUCTION OF PROTEINS INVOLVED IN XENOBIOTIC METABOLISM

The present application is a Continuation-in-Part of United States patent application serial number 10/222,679 entitled "Compositions and Methods for Induction of Proteins Involved in Xenobiotic Metabolism", filed August 16, 2002, and is a Continuation-in-Part of United States patent application serial number 09/832,621 entitled "Compositions and Methods for Induction of Proteins Involved in Xenobiotic Metabolism", filed April 11, 2001, and claims benefit of priority to United States provisional patent application serial number 60/241,391 entitled "High Volume Screening for p450 Induction" filed October 17, 2000, and United States provisional patent application serial number 60/196,681 entitled "Method for In Vitro Screening for Drug Metabolism" filed on April 12, 2000, each of which is incorporated by reference herein in their entirety.

This invention was made with government support awarded by the National Institutes of Health, grant number GM-58287. The United States Government may have certain rights in the invention.

Technical Field

The invention relates to the field of identifying compounds that alter expression of proteins.

Background

Adverse reactions to therapeutic agents are a common cause of morbidity and mortality, particularly in industrialized nations where the use of such therapeutic agents is relatively common. It has been estimated that side affects from drugs are the fourth to sixth leading cause of death in hospitals in the United States (Moore and Kliewer (2000) *Toxicology,* 153:1-10). A large number of these adverse reactions are due to drug interactions, a process by which the

administration of one drug alters the properties of a second co-administered drug. The most common drug interactions occur when one drug either increases or decreases the effectiveness of another (Moore and Kliewer (2000) *Toxicology,* 153:1-10). This modification in the pharmacological action of a drug generally stems from alternations in the drug's metabolism. Thus, a major factor associated with drug interactions is altered metabolism.

The tissues most relevant to drug metabolism are the liver and intestine. Within these tissues the oxidative metabolism of drugs and other xenobiotics occurs through the action of a super-family of heme containing monooxygenases, collectively known as cytochrome P450 enzymes (CYP's) (Nebert and Gonzalez (1987) *Ann. Rev. Biochem.,* 56:945-993). In general, the enzymatic actions of CYPs results in the formation of products with greater polarity, causing more rapid elimination of the product relative to the drug itself. This process can significantly alter a drug's pharmacodynamic profile. Such reactions are particularly important when they affect drugs with narrow therapeutic ranges.

The most abundant CYP enzyme present in the human liver and intestine is CYP3A4, accounting for about 70% of total enterocyte CYPs (Moore and Kliewer (2000) *Toxicology,* 153:1-10) and about 29% to about 60% of hepatic P450s (Wrighton *et al.* (2000) *Drug Metab. Rev,.* 32:339-361). Substrates for CYP3A4, a microsomal enzyme, are generally highly lipophilic. The structural divergence of known CYP3A4 substrates is wide and includes endogenous steroids, contraceptive steroids, immunosuppressive agents, imidazole antimycotics and macrolide antibiotics (Wrighton *et al.* (2000) *Drug Metab. Rev.,* 32:339-361). Because of the abundance of CYP3A4 in liver and intestine and its broad substrate specificity, CYP3A4 is believed to play a dominant role in drug biotransformation. It is estimated that this P450 enzyme is involved in the metabolism of greater than 50% of all drugs in use today (Wrighton *et al.* (2000) *Drug Metab. Rev,.* 32:339-361).

Prolonged exposure to drugs can lead to an increased expression of specific p450s that can augment the metabolism and clearance of therapeutic drugs. CYP3A4 activity is enhanced by a range of diverse chemicals and its induced expression is the cause of many drug interactions. Several of the most efficacious inducers of CYP3A4 expression are commonly used drugs such as the gluccocorticoid dexamethasone, the anticonvulsant phenobarbital, the antibiotic rifampicin

and the antimycotic clotrimazone (Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023). As a result of elevated CYP3A4 levels, therapeutics metabolized by this P450 exhibit lower efficacy. Therefore, it is important to identify agents possessing the ability to induce drug-metabolizing enzymes including, but not limited to, CYP3A4 and other p450 enzymes.

The biochemistry of P450 regulation can be complex. Some inducers of p450 activity have been identified. The levels of CYP3A4 are induced by exposure to a number of structurally diverse agents. This diversity can make it difficult to predict new drugs that may affect expression of that enzyme. Glucocorticoids and other nonsteroidal inducers of CYP3A4 may transcriptionally regulate the expression of this P450 by a mechanism involving an orphan nuclear receptor, pregnane X receptor (PXR) and potentially other receptors. PXR was identified as a new member of the nuclear hormone receptor super family. PXR mediates high dose glucocorticoid and pregnane steroid induction of the CYP3A4 promoter by heterodimerizing with the nuclear hormone receptor partner RXR and binding to an element highly conserved in the CYP3A4 promoter, the PXR element (PXRE). The nucleotide constraints for PXR binding have apparently been defined as AGTTCA arranged as a direct repeat (DR) or everted repeat (ER) with three, four, five or six nucleotide spacing (Wrighton *et al.* (2000) *Drug Metab. Rev.*, 32:339-361).

Because CYP3A4 and other CYPs can exhibit species differences, pharmaceutical companies test their drug candidates in vitro in human systems in order to gain an assessment of the potential for drug interactions in humans. Most in vitro testing involves the use of primary cultures of human hepatocytes. The availability of hepatocytes has afforded the pharmaceutical industry the ability to obtain clinically relevant in vitro drug interaction data. Compounds that are identified as potential inducers of a human P450 in hepatocytes can be screened out of further development, helping to alleviate the potential for a drug interaction and hence a safety and marketing liability (Rodrigues (1997) *Pharm. Res.*, 14:1504-1510).

There are, however, disadvantages to utilizing primary cultures for these tests. One logistical problem with hepatocyte preparations is that enzymatic activities are not stable for longer than about four or five days. Also, these systems are costly, time consuming and produce variable responses. Furthermore, availability can be sporadic because primary cultures rely on

the availability of human organs. Results obtained using these cells are also dependent upon culture media and conditions of culture. Finally, a limited number of compounds can be tested at any given time. This is particularly problematic because large numbers of candidate drugs are being produced through combinatorial chemistry and combinatorial biology methods.

Because of the inherent problems associated with the use of human hepatocytes for preclinical drug development, and the difficultly in obtaining liver specimens for research purposes, other in vitro systems are being investigated. Transcriptional activation has been performed in vitro for a number of years to investigate changes in gene expression of P450 enzymes by chemicals (Plant *et al.* (2000) *Anal. Biochem.*, 278:170-174). The most common type is to use transient transfections of a reporter gene construct into a suitable cell line. This is then followed by dosing with a test compound, measuring of reporter gene production and comparison to control cells. At each step of this protocol, biological and experimental variations may be present which can provide poorly reproducible results and potentially erroneous interpretations. Examples of such variations include initial transfection efficiency, activation by factors endogenous to the host cell line and chemical specific effects such as cytotoxicity or proliferative effects. These problems decrease enthusiasm for using these types of systems.

Brief Description of the Figures

FIG. 1 depicts a series of figures for one aspect of the present invention, where the first nucleic acid molecule and second nucleic acid molecule are provided as extra chromosomal elements such as plasmids. As depicted in **FIG. 1A**, a regulatory element P2 modulates the transcription of the gene encoding an intracellular receptor or transcription factor. The translation product can then interact with a test compound that binds with the intracellular receptor or transcription factor. As depicted in **FIG. 1B**, the complex of the intracellular receptor or transcription factor and xenobiotic or test compound can then bind with the promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism. The complex can also enter the nucleus and optionally bind with the endogenous promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism, if present or active in such cell. Upon binding of this complex with the promoter or enhancer

operable for a nucleic acid molecule encoding a protein involved in drug metabolism, a reporter gene is transcribed and translated into a reporter and optionally the endogenous enzyme involved in drug metabolism is expressed, if present or active in such cell (**FIG. 1C**). That reporter can be detectable by its physical properties, such as fluorescence or luminescence, or can be a protein that is detectable based on its enzymatic conversion of substrate to product, such as a detectable product (**FIG. 1D**). Such reporters can be intracellular or extracellular. In another aspect of the present invention, both the first nucleic acid molecule and the second nucleic acid molecule are provided on the same extra chromosomal element, such as a single plasmid or YAC.

FIG. 2 depicts the case where the first nucleic acid molecule is an extra chromosomal element (**20**) whereas the second nucleic acid molecule is endogenous to the chromosome of the cell (**22**).

FIG. 3 depicts the case where the first nucleic acid molecule is an extra chromosomal element (**30**) and the second nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell (**32**).

FIG. 4 depicts the case where the first nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell (**40**) and the second nucleic acid molecule is endogenous to the chromosome of the cell (**42**).

FIG. 5 depicts the case where the first nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell (**50**) and the second nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell (**52**).

FIG. 6 depicts the construction of a HepG2 cell line containing the stably integrated CYP3A4 PXRE/luciferase reporter construct. The CYP3A4 PXRE containing the ER6 DNA sequence is provided in plasmid pGL3 Promoter (Promega). The transfected cells are cultured in the presence of geneticin to select for the integrated plasmid pIRESneo (Clonetech). Geneticin resistant colonys are screened for rifampicin enhanced light emission.

FIG. 7 depicts the response of the HepG2 cell line depicted in **FIG. 6**. Upon binding to a ligand such as a new chemical entity (NCE), endogenous HepG2 PXR is activated and forms a heterodimer with endogenous HepG2 RXR. The PXR/RXR complex binds to the PXRE sequence that was cloned into pGL3-Promoter (Promega) and stably integrated into the HepG2

genome. Binding of the PXR/RXR complex activates transcription from the SV40 promoter of the integrated pGL3 Promoter plasmid. The luciferase gene is transcribed and translated causing the NCE dose-dependent emission of light.

FIG. 8 depicts a Northern blot analysis of RNA isolated from individual colonies stably transformed with pIRES containing hPXR alone (colonies D6, D4, B6, B5, A5, A2, W6, W5, W4, W2 and W1) or in combination of pGL3 promoter containing the CYP3A4 enhancer element described in the Examples (colony 6G). Each well contains 10 micrograms of total RNA and was developed with a cDNA probe specific for hPXR.

FIG. 9 depicts the effect of rifampicin and DMSO treatment on cells stably transformed with pGL3-promoter/3A4 enhancer. A 96 well plate was used to determine the length of exposure to produce high levels of induction of luciferase. Cells were treated between zero and seventy-eight hours prior to measuring luciferase activity. Results are expressed as fold increase over DMSO control cells and are the results of quadruplicate experiments.

FIG. 10 depicts the effect of rifampicin treatment on cells containing pGL3/3A4 enhancer plus phPXR. Cells were plated in a 96 well plate format and exposed to 10 micromolar rifampicin or DMSO for seventy-two hours. Results are expressed as relative light units and are the result of quadruplicate experiments.

FIG. 11 depicts various amounts of cells containing either hPXR plus pGL3/3A4 enhancer or the pGL3/3A4 enhancer alone were added to a 96 well plate and treated with 10 micromolar rifampicin or DMSO for forty-eight hours. Results are expressed as fold increase above control DMSO treated cells and are the result of quadruplicate experiments.

FIG. 12 depicts the effect of serum, DMSO and rifampicin on luciferase activity in HepG2 cells stably transformed with the pGL3 vector and pIRES vector with hPXR. Cells were treated for various time periods ranging from zero to seventy-eight hours in the presence or absence of rifampicin, DMSO or 0.1% serum in the media. An additional control without either rifampicin or DMSO was also included. Results are expressed as relative light units and are the result of quadruplicate experiments.

FIG. 13 depicts the effect of various CYP3A4 inducers on CYP3A4 expression in human hepatocytes. Human hepatocytes were exposed to 10 micromolar dexamethasone, no

dexamethasone or the amount of dexamethasone normally present in hepatocyte culture media (about 10^{-7} M). Other inducers include one millimolar phenobarbital, ten micromolar rifampicin, clotrimazole or RU486. Total RNA (ten micrograms) was subjected to northern blot analysis and developed with a specific cDNA probe to CYP3A4 as described in the Examples.

FIG. 14 depicts the effects of various CYP3A4 inducers and non-inducers on HepG2 cells stably transformed with hPXR in pIRES vector and the 3A4 enhancer in the luciferase vector (colony 1F). Cells were exposed to each inducer for seventy-two hours in a 96 well plate format prior to determining luciferase activity. Cells were treated with one micromolar dexamethasone, one hundred micromolar omeprazole, ten micromolar clotrimazole, ten micromolar RU486, ten micromolar rifampicin, one hundred micromolar mevastatin, fifty micromolar PCN, one hundred micromolar phenobarbital, one micromolar TCDD. Data is expressed as fold increase in luciferase activity above that in control DMSO treated cells and represents quadruplicate determinations.

FIG. 15 depicts the effects of various CYP3A4 inducers and non-inducers on HepG2 cells stably transformed with the 3A4 enhancer in the luciferase vector (colony 13). Cells were exposed to each inducer for seventy two hours in a 96 well plate format prior to determining luciferase activity. Cells were treated with one micromolar dexamethasone, one hundred micromolar omeprazole, ten micromolar clotrimazole, ten micromolar RU486, ten micromolar rifampicin, one hundred micromolar mevastatin, fifty micromolar PCN, one hundred micromolar phenobarbital, one micromolar TCDD. Data is expressed as fold increase in luciferase activity above that in control DMSO treated cells and represents quadruplicate determinations.

FIG. 16 depicts the effects of various doses of different CYP inducers on HepG2 cells stably transformed with the CYP3A4-enhancer in the luciferase vector (colony 13). Cells were exposed to each inducer for seventy two hours in a 96-well plate format prior to determining luciferase activity. Cells were treated with three doses of each drug. Doses ranged from 0.1 micromolar to 5 millimolar, depending on the agent. For dexamethasone doses were 0.1 micromolar, 1.0 micromolar and 10 micromolar; for omeprazole 50 micromolar, 100 micromolar and 250 micromolar; for clotrimazole 5 micromolar, 10 micromolar and 50 micromolar; for phenobarbital 1 millimolar, 2 millimolar and 5 millimolar; for TCDD 0.5 nanomolar, 1

nanomolar and 2 nanomolar; for RU486 5 micromolar, 10 micromolar and 50 micromolar; for rifampicin 5 micromolar, 10 micromolar and 25 micromolar; and for mevastatin 10 micromolar, 50 micromolar and 100 micromolar. Results are expressed as fold increase in luciferase activity above DMSO-treated cells and are the mean +/- standard deviation of six determinations. The lowest dose of each drug is represented as increasing from left to right.

FIG. 17 depicts the effects of plating stable cell lines in 24 and 96 well plates. 101L cells were plated in 24 or 96 well plates and exposed to various doses of the Ah receptor ligand benzanthracene. After 18 hour exposures, luciferase activity was assessed. Results are expressed as the mean of three different experiments +/- SD.

FIG. 18 depicts a time response curve of various CYP1A1 inducers. The maximal time period for inducer exposure was determined by establishing a time course of inducer mediated luciferase activity in 101L cells and with the 96 well plates. Enhanced activity was observed within 6 hours of dosing with benzanthracene (100 micromolar), omeprazole (100 micromolar) and 3-methylcholanthrene (10 micromolar). Cells were also treated with rifampincin (100 micromolar) as a negative control. Each point represents the mean of results from three experiments +/- SD.

FIG. 19 depicts dose response curve of various known CYP1A1 inducers. The effects of various CYP1A1 inducers were determined using 96 well plate format and the 101L cells. Dose response curves were generated to TCDD (0.5 to 2.2 nanomolar (panel A), benzanthracene and omeprazole (1 to 200 micromolar) (panel B). Each point represents the mean of results from three experiments +/- SD.

FIG. 20 depicts dose response curves for various flavonoids. Using the 96 well plate format and the 101L cells, dose response curves were generated for GTE (inset). Doses ranged from 0.01 milligrams/ml to 0.2 milligrams/ml and 18 hours of exposure. Dose response curves were also determined for EGCG, quercetin, curcumin, kaempferol, naringenin, apigenin, and resveratrol and ranged from 1 to 20 micromolar. Exposure to each agent was for 18 hours. Each point represents the mean of results from three experiments +/- SD.

FIG. 21 depicts the effects of co-treatment with TCDD and each flavonoid. The CYP1A1 containing cell line was treated with 10 micromolar of each flavonoid or 0.1

milligrams/ml of GTE and 2 nanomoles of TCDD. Cells were exposed to both agents for 18 hours. Results represent the mean of three experiments +/- SD.

FIG. 22 depicts distal promoter elements (**SEQ ID NO. 15, 35, 36, 37**), identified in CYP2C19, CYP2C9, CYP3A4, and CYP2B6, respectively, which are believed to bind nuclear receptors and to activate transcription via enhancer mechanisms as described in Example 4. NR1 sequences from all 3 genes are shown in bold type. Spacers (shown as dots) are inserted to visually align the receptor binding half-sites from the different genes.

FIG. 23 depicts Northern Blot analyses of the effect of various test compounds on CYP1A1 expression in human hepatocytes as described in Example 5. **FIG. 23A:** Human hepatocytes were exposed for 24 hours to 0.1% of dimethylsulfoxide (DMSO) as a vehicle, or to the environmental contaminants dimethylbenzo(a)pyrene (DMBA) (20 micromoles per liter), 2-acetylaminofluorene (2-AAF) (10 and 100 micromoles per liter), or 3-methylcholanthrene (3-MC) (0.1 and 0.5 nanomoles per liter), **FIG. 23B:** Human hepatocytes were exposed for 24 hours to the therapeutic agent omeprazole (100 micromoles per liter), to the plant flavonoids chrysin (25 micromoles per liter), curcumin (50 micromoles per liter), and resveratrol (25 micromoles per liter), or to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (0.5 and 10 nanomoles per liter). The isolated RNA was subjected to Northern blot analysis. The RNA blot was hybridized to a ^{32}P-labeled CYP1A1 cDNA probe (Allen *et al.* (2001) *Drug Metab. Disp.*, 29:1074-1079) (top panel) or to a ^{32}P-labeled 18s rRNA probe for normalization of RNA loading on the gel (bottom panel).

FIG. 24 depicts the time-course and dose-response relationship of luciferase expression in DRE12-6 cells, which are human hepatoma HepG2 cells stably transfected with a luciferase reporter plasmid harboring multiple dioxin response elements. **FIG. 24A:** DRE12-6 cells were grown in a 96-well plate and treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (10 nanomoles per liter) at 4, 20, 24, and 28 hour time points. The maximum induction was obtained at the 28 hour time point where TCDD treatment resulted in a 20-fold induction of luciferase activity. **FIG. 24B:** DRE12-6 cells were also treated with varying concentrations of TCDD and 3-methylcholanthrene (3-MC) to obtain a dose-response profile of luciferase induction. Cells were lysed after chemical exposure and analyzed for luciferase activity with Luclite kit (Packard

Instrument Co., Meriden, CT). Results are depicted as the fold induction of luciferase activity above the 0.1% dimethylsulfoxide (DMSO) vehicle control. Values are the mean ± SD of 3 determinations.

FIG. 25 depicts the ability of natural products (herbal constituents) to activate AhR-mediated luciferase induction in DRE12-6 cells. **FIG. 25A:** DRE12-6 cells were treated for 28 hours with the indicated concentrations of phytochemicals. **FIG. 25B:** DRE12-6 cells were treated with the indicated chemicals for 3 hours prior to co-treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 28 hours.

Chemical treatment was followed by cell lysis and luciferase measurement. Results are depicted as the fold induction of luciferase activity above the 0.1% DMSO vehicle control. Values are the mean ± SD of 3 determinations.

FIG. 26 depicts results of testing environmental contaminants for their ability to activate AhR-mediated luciferase induction in DRE12-6 cells. **FIG. 26A:** DRE12-6 cells were inoculated into a 96-well plate (2×10^4 cells per well) a day prior to treatment and treated for 28 hours with the indicated concentrations of the environmental contaminants (PAHs, PAH derivatives, PCBs, or pesticide derivatives). **FIG. 26B:** DRE12-6 cells were treated with the indicated chemicals for 3 hours prior to co-treatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) for 28 hours.

Chemical treatment was followed by cell lysis and luciferase measurement. Results are depicted as the fold induction of luciferase activity above the 0.1% dimethylsulfoxide (DMSO) vehicle control. Values are the mean ± SD of 3 determinations.

FIG. 27 depicts the inhibition of TCDD-mediated CYP1A1 induction by green tea extract as demonstrated by Northern Blot Analysis. DRE12-6 cells were treated with the vehicle dimethylsulfoxide (DMSO), green tea extract (GTE), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or a combination of TCDD and GTE, for either 6 hours or 18 hours as indicated. Total RNA was prepared from the treated cells, subjected to electrophoresis on a 1% formaldehyde gel, and transferred onto a nylon membrane. The RNA blot was hybridized to a ^{32}P-labeled CYP1A1 cDNA probe (Allen *et al.* (2001) *Drug Metab. Disp.,* 29:1074-1079) (top panel) or to a ^{32}P-labeled 18s rRNA probe for normalization of RNA loading on the gel (bottom panel).

FIG. 28 depicts CYP3A4 gene expression in response to various chemicals, including plant flavonoids, as shown by Northern blot analysis. **FIG. 28A:** Human primary hepatocytes were exposed for 48 hours to either 0.1% dimethylsulfoxide (DMSO), or to the test compounds apigenin, resveratrol, curcumin, quercetin, and beta-naphthoflavone (BNF) as indicated. Rifampicin (Rif), omeprazole, and phenobarbitol are known PXR ligands and were used as positive controls for CYPE3A4 induction. **FIG. 28B:** Human primary hepatocytes were exposed for 48 hours to commercial health supplements including grapeseed, garlic, kava-kava, and ginseng.

Total RNA was prepared from the treated cells, subjected to electrophoresis on a 1% formaldehyde gel, and transferred onto a nylon membrane. The RNA blot was hybridized to a ^{32}P-labeled CYP1A1 cDNA probe (Allen *et al.* (2001) *Drug Metab. Disp.*, 29:1074-1079) (top panel) or to a ^{32}P-labeled 18s rRNA probe for normalization of RNA loading on the gel (bottom panel).

FIG. 29 depicts CYP3A4 gene regulation in response to PXR ligands by the stably transfected cell line Hepg-PXR (HepG2 cells overexpressing PXR). Total RNA was prepared from Hepg-PXR cells chemically treated as indicated. The RNA blot was hybridized with ^{32}P-labeled cDNA for CYP3A4 (upper panel). Ethidium bromide staining of the blot demonstrated that equal amounts of total RNA were analyzed (lower panel).

FIG. 30 depicts the results of testing various compounds for their ability to induce PXR-mediated CYP3A4 expression in the stable cell line DPX2 (Hepg-hPXR/3A4-Luc), as indicated by luciferase activity. Compounds tested included known PXR ligands and conventional drugs (**FIG. 30A**), environmental contaminants (**FIG. 30B**), and herbal supplements (**FIG. 30C**). DPX2 cells were cultured with DMEM supplemented with 10% fetal bovine serum and neomycin. Cells were inoculated into 96-well plate at a density of 1 X 10^4 cells per well the day before treatment. Abbreviations used: pregnenolone 16 alpha-carbonitrile (PCN), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2-acetylaminofluorene (2-AAF), and 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorbenzyl)oxime (CITCO).

After 48 hours treatment with test compounds, cells were lysed after chemical exposure and analyzed directly in the 96-well plate for luciferase activity with a Luclite system (Packard

Instrument Co., Meriden, CT). Results are depicted as the fold induction of luciferase activity above the 0.1% dimethylsulfoxide (DMSO) vehicle control. Values are the mean ± SD of 3 determinations.

FIG. 31 depicts *MDR1* gene expression in LS174T cells as analyzed by Northern blot. Cells were treated for 48 hours with rifampicin (RIF), omeprazole (omp), mifepristone (Mif), dexamethasone (Dex), pregnenolone 16-alpha-carbonitrile (PCN) at the indicated concentrations (all in micromoles per liter). Total RNA from the colon adenocarcinoma cell line LS174T was isolated and fractionated on a 1% formaldehyde gel and transferred onto a nylon membrane. The RNA blot was hybridized to ^{32}P-labeled *MDR1* cDNA probe (top panel) or 18s rRNA probe for normalization of RNA loading on the gel (bottom panel).

FIG. 32 depicts *MDR1* and *CYP3A4* expression profiles in response to PXR activator rifampicin as studied by Northern blot. **FIG. 32A:** Lanes 1 and 2, primary human hepatocytes; lanes 3 and 4, LS174T cells. **FIG. 32B:** Lanes 1 and 2, HepG2 cells; lanes 3 and 4, HepG2 cells that overexpress PXR (Hepg-PXR cells). **FIG. 32C:** Lanes 1 and 2, Caco-2; lanes 3 and 4, Caco-2 cells that overexpress PXR (Caco-PXR cells).

Cells were exposed to dimethylsulfoxide (DMSO) as a vehicle control or to rifampicin (Rif) (10 micromoles per liter) for 48 hours. Total RNA from the colon adenocarcinoma cell line LS174T was isolated and fractionated on a 1% formaldehyde gel and transferred onto a nylon membrane. The RNA blot was hybridized to ^{32}P-labeled *MDR1* cDNA probe (top panels), ^{32}P-labeled *CYP3A4* cDNA probe (middle panels), or 18s rRNA probe for normalization of RNA loading on the gel (bottom panels).

FIG. 33 depicts RNA transcripts of human PXR, compared by mRNA concentration-dependent reverse transcription. Conditions were as described in Example 6. **FIG. 33A:** Total RNA from Caco-2/PXR, Caco-2, HepG2/PXR, HepG2, and LS174T cells, and from human primary hepatocytes, was isolated and used in the amounts indicated as a template for reverse transcription-PCR. Top panel shows a negative image of the agarose gel under UV transillumination with a molecular weight ladder in the rightmost lane. The bottom panel shows ethidium bromide staining of the agarose gel to demonstrate integrity of the RNA and that approximately the same amounts of RNA were used for each cell type. **FIG. 33B:** The

response of endogenous PXR levels to PXR ligands were examined in LS174T cells by RT-PCR. LS174T cells were exposed for 48 hours to pregnenolone 16-alpha-carbonitrile (PCN), rifampicin, or dimethylsulfoxide (DMSO, control) at the concentrations indicated. The figure shows a negative image of the agarose gel under UV transillumination with a molecular weight ladder in the outermost lanes.

FIG. 34 depicts mdr1(dr4) response element and PXR-dependent transcriptional activation behavior, characterized by transient transfection experiments as described in Example 6. Luciferase activity is expressed as relative light units (RLU). Each assay was conducted in triplicate and results are shown as means ± SD. **FIG. 34A:** Results of transcriptional activation by human PXR ligands in transfected Caco-2 are shown. PXR co-transfected with PGL3P, or CAR co-transfected with pgp-DR4, were used as negative controls. **FIG. 34B:** Results of treatment of mdr1(dr4) and PXR co-transfected HepG2 cells at the indicated concentrations of rifampicin (RIF) are shown. A dose-dependent increase of relative luciferase activity was observed.

FIG. 35 depicts dose-response behavior to various PXR activators in stable cell lines overexpressing PXR and harboring DR4 response element. **FIG. 35A:** Results are shown for the stable cell line Hep-PXR/PgpLuc. **FIG. 35B:** Results are shown for the stable cell line Caco-PXR/PgpLuc.

Cells were exposed to the test compounds for 48 hours at the concentrations indicated. This was followed by cell lysis and luciferase measurement. Results are depicted as the fold induction of luciferase activity above the 0.1% DMSO vehicle control. Values are the mean ± SD of 3 determinations.

FIG. 36 depicts the effect of environmental and botanical chemicals on luciferase activity in the stable cell line Hep-PXR/PgpLuc. Cells were inoculated into 96-well plates at a density of 2×10^4 cells per well. After overnight recovery, cells were treated with the test compounds at the concentrations indicated for 48 hours. 2AAF is 2-acetylaminofluorene; GTE is green tea extract. The known PXR ligand, rifampicin, was used to obtain a dose-response curve as a positive control. This was followed by cell lysis and luciferase measurement. Results are depicted as the fold induction of luciferase activity above the 0.1% DMSO vehicle control.

Values are the mean ± SD of 3 determinations.

FIG. 37 depicts *MDR1* gene regulation in response to chrysin as studied by Northern blot analysis. Human primary hepatocytes were exposed to 0.1% dimethylsulfoxide (DMSO) as a vehicle control, to the known PXR ligand rifampicin (Rif) (10 micromoles per liter), or to the flavonoid chrysin (25 micromoles per liter) for 48 hours. Total RNA from the hepatocytes was isolated and fractionated on a 1% formaldehyde gel and transferred onto a nylon membrane. The RNA blot was hybridized to 32P-labeled *MDR1* cDNA probe (top panels), ^{32}P-labeled *CYP3A4* cDNA probe (middle panels), or 18s rRNA probe for normalization of RNA loading on the gel (bottom panels).

Summary

The *in vitro* system described herein can detect induction of drug metabolizing enzymes, including P450s such as CYP3A4. The disclosed methods can detect transcriptional activation by xenobiotics of an appropriate enhancer and reporter gene that have been optionally independently stably transfected into a host cell, such as human hepatoma cells. The system can be utilized in a microtiter plate format and results can optionally be obtained with an appropriate microtiter plate reader within two or three days of drug candidate application to the cells. The advantages of this *in vitro* transcription system as compared to isolated human hepatocytes or liver slices are numerous, including increased consistency and reproducibility of the assay. Also, inter-individual or inter-sample variability and culture conditions that can influence the results of an assay are addressed using the systems of the present invention. The present system can be formatted for high throughput assays and can predict a two-fold or greater induction of a specific drug metabolizing protein encoding gene in a relatively short time period.

In one preferred aspect of the present invention, the *in vitro* system is high throughput in nature and can assess CYP3A4 induction. This preferred aspect of the present invention includes the regulatory region of the CYP3A4 gene named the PXRE and the transcription factor PXR. The PXRE is operably linked to a reporter gene such as luciferase, such as on a plasmid. The plasmid containing the PXRE and reporter gene is then stably transformed into a hepatoma cell

line, such as HepG2. Once transformed, the PXR can bind to the PXRE and activate transcription. This can occur when the PXR is stimulated by an appropriate ligand, such as a drug. A nucleic acid molecule encoding a drug metabolizing protein other than CYP3A4 or other than P450's can be used by substituting nucleic acid molecules. Appropriate regulatory regions other than PXRE can also be used, such that the regulatory region is appropriate for the nucleic acid molecule encoding a drug metabolizing enzyme or transporter. In addition, reporter genes other than luciferase, such as detectable proteins, such as Green Fluorescent Protein (GFP) or its variations, or other enzymes, such as beta-galactosidase, beta-lactamase or alkaline phosphatase can be used in this system. Alternative cells can be used, but cells that are derived from tissues involved in drug metabolism are preferred.

The present invention recognizes that cell based systems for evaluating compound interactions can be made using appropriate nucleic acid molecules that include one or more enhancers or promoters for a nucleic acid molecule encoding a protein involved in drug metabolism operably linked to a nucleic acid molecule encoding a reporter gene, and a nucleic acid molecule encoding an intracellular receptor or transcription factor. These nucleic acid molecules can be extra chromosomally or stably integrated into the genome of a cell. In addition, in certain cases the nucleic acid molecules can be endogenous to the chromosome of the cell, particularly in the case where the nucleic acid molecule encodes an intracellular receptor, transporter or transcription factor.

One aspect of the present invention provides a cell that includes a first nucleic acid molecule that includes: a promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism and a reporter gene. Preferably, the promoter or enhancer is operably linked to said reporter gene. The cell also includes a second nucleic acid encoding an intracellular receptor or transcription factor, such that when the intracellular receptor or transcription factor is bound or activated with a compound, said intracellular receptor or transcription factor can operably bind with said promoter or enhancer resulting in the expression of said reporter gene. When the cell is contacted with a compound that induces the expression of the protein involved in drug metabolism, the reporter gene is expressed.

A second aspect of the present invention provides a method for evaluating compounds for the property of inducing the expression of a gene encoding a protein involved in drug metabolism, including; providing a test compound; contacting the test compound with a cell of the present invention; and detecting the expression of said reporter gene. The expression of the reporter gene is indicative that said compound altered the expression of a gene encoding a protein involved in drug metabolism.

Detailed Description of the Invention

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, chemistry, microbiology, molecular biology, cell science and cell culture described below are well known and commonly employed in the art. Conventional methods are used for these procedures, such as those provided in the art and various general references (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1989); Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley and Sons (1998); Harlowe and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor Press (1988)). Where a term is provided in the singular, the inventors also contemplate the plural of that term. The nomenclature used herein and the laboratory procedures described below are those well known and commonly employed in the art. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

A "nucleic acid molecule" is a polynucleotide. A nucleic acid molecule can be DNA, RNA, or a combination of both. A nucleic acid molecule can also include sugars other than ribose and deoxyribose incorporated into the backbone, and thus can be other than DNA or RNA. A nucleic acid can comprise nucleobases that are naturally occurring or that do not occur in

nature, such as xanthine, derivatives of nucleobases such as 2-aminoadenine and the like. A nucleic acid molecule of the present invention can have linkages other than phosphodiester linkages. A nucleic acid molecule can also be a peptide nucleic acid molecule. A nucleic acid molecule can be of any length, and can be single-stranded or double-stranded, or partially single-stranded and partially double-stranded.

A "probe" or "probe nucleic acid molecule" is a nucleic acid molecule that is at least partially single-stranded, and that is at least partially complementary, or at least partially substantially complementary, to a sequence of interest. A probe can be RNA, DNA, or a combination of both RNA and DNA. It is also within the scope of the present invention to have probe nucleic acid molecules comprising nucleic acids in which the backbone sugar is other than ribose or deoxyribose. Probe nucleic acids can also be peptide nucleic acids. A probe can comprise nucleolytic-activity resistant linkages or detectable labels, and can be operably linked to other moieties, for example a peptide.

A single-stranded nucleic acid molecule is "complementary" to another single-stranded nucleic acid molecule when it can base-pair (hybridize) with all or a portion of the other nucleic acid molecule to form a double helix (double-stranded nucleic acid molecule), based on the ability of guanine (G) to base pair with cytosine (C) and adenine (A) to base pair with thymine (T) or uridine (U). For example, the nucleotide sequence 5'-TATAC-3' is complementary to the nucleotide sequence 5'-GTATA-3'.

"Substantially complementary" refers to nucleic acids that will selectively hybridize to one another under stringent conditions.

"Selectively hybridize" refers to detectable specific binding. Polynucleotides, oligonucleotides and fragments thereof selectively hybridize to target nucleic acid strands, under hybridization and wash conditions that minimize appreciable amounts of detectable binding to nonspecific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art. Generally, the nucleic acid sequence complementarity between the polynucleotides, oligonucleotides, and fragments thereof and a nucleic acid sequence of interest will be at least 30%, and more typically and preferably of at least 40%, 50%, 60%, 70%, 80%, 90%, and can be 100%. Conditions for hybridization such as

salt concentration, temperature, detergents, and denaturing agents such as formamide can be varied to increase the stringency of hybridization, that is, the requirement for exact matches of C to base pair with G, and A to base pair with T or U, along the strand of nucleic acid.

"Corresponds to" refers to a polynucleotide sequence that shares identity (for example is identical) to all or a portion of a reference polynucleotide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence will base pair with all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence 5'-TATAC-3' corresponds to a reference sequence 5'-TATAC-3' and is complementary to a reference sequence 5'-GTATA-3'.

"Sequence identity" or "identical" means that two polynucleotide sequences are identical (for example, on a nucleotide-by-nucleotide basis) over the window of comparison. "Partial sequence identity" or "partial identity" means that a portion of the sequence of a nucleic acid molecule is identical to at least a portion of the sequence of another nucleic acid molecule.

"Substantial identity" or "substantially identical" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 30 percent sequence identity, preferably at least 50 to 60 percent sequence identity, more usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25 to 50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence that may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. "Substantial partial sequence identity" or "substantially partially identical" is used when a portion of a nucleic acid molecule is substantially identical to at least a portion of another nucleic acid molecule. As used herein "identity" or "identical" refers to the base composition of nucleic acids, and not to the composition of other components, such as the backbone that can be comprised of one or more sugars and one or more phosphates, or can have other substituted moieties.

A "mutation" is a change in the genome with respect to the standard wild-type sequence. Mutations can be deletions, insertions, or rearrangements of nucleic acid sequences at a position in the genome, or they can be single base changes at a position in the genome, referred to as

"point mutations". Mutations can be inherited, or they can occur in one or more cells during the lifespan of an individual.

"Hybridization" is the process of base-pairing of single-stranded nucleic acids, or single-stranded portions of nucleic acids, to create double-stranded nucleic acids or double-stranded portions of nucleic acid molecules.

A "single nucleotide polymorphism" or "SNP" is a position in a nucleic acid sequence that differs in base composition in nucleic acids isolated from different individuals of the same species.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting them to function in their intended manner. For example, a control sequence such as promoter or enhancer or other regulatory sequence operably linked to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with control sequences.

"Operable" in the sense of a control sequence being operable for a nucleic acid molecule encoding a polypeptide or protein, such as a protein involved in drug metabolism, refers to the ability of the control sequence to regulate the expression of such polypeptide or protein under appropriate configurations, such as being operably linked and under appropriate conditions, such as binding of appropriate modulators in appropriate configurations to the control sequence.

A "promoter" refers to a nucleic acid molecule, such as in DNA, to which RNA polymerase binds to begin transcription. A promoter can be considered a component of the gene control region where the transcription factors and the polymerase assemble to control transcription.

An "enhancer" refers to regulatory nucleic acid molecules, such as DNA sequences, to which gene regulatory proteins bind, which can influence the rate of trancription of a structural gene. Examples of enhancers include GAL4 protein attaching to a regulatory region of the LacZ gene to influence expression of beta-galactosidase. Another example is the pregnane X receptor (PXR) which binds to a DNA sequence termed PXRE or XREM that regulates the rate of transcription of the enzyme CYP3A4. A further example is the Ah receptor (AhR), which binds

to specific DNA sequences termed DRE, the dioxin response element, in the regulatory region of CYPs 1A1 and 1A2 to regulate the rate of transcription.

A "protein involved in drug metabolism" refers to a protein or polypeptide, such as a protein, that is capable of metabolism or modulating the metabolism of a xenobiotic such as a drug. Such modulating includes changing the chemical structure of the xenobiotic through catalytic reactions and covalent or non-covalent bonds, altering the permeability of a xenobiotic into or out of a cell, or transporting a xenobiotic into or out of a cell.

A "drug metabolizing enzyme" refers to enzyme proteins that catalyze the covalent modification of xenobiotics such as drugs that are foreign to the host. Such covalent modifications can be any, but are preferably oxidation or conjugation reactions. The oxidation reactions generally result in water soluble metabolites or metabolites with increased water solubility. For example CYP3A4 metabolizes the drug erythromycin to a demethylated metabolite, increasing its polarity. Glucuronosyltransferase 1 (UGT1) adds a glucuronide to acetaminophen to increase its polarity. CYP2C19 metabolizes S-mephenytoin by adding an hydroxyl group to the anticonvulsant. Generally, by increasing the polarity of the xenobiotic, the modified xenobiotic is more readily eliminated from the subject, such as through the urine.

A "reporter gene" refers to a region of a nucleic acid molecule such as DNA that encodes a protein that is readily detected by an assay. This region can replace the normal coding region of a gene. For example, the luciferase gene encodes the luciferase protein that can produce luminescent products can be detected by a luminometer. The LacZ gene encodes the beta-galactosidase protein that can convert certain substrates to colored forms that can be detected colormetrically or fluorimetrically in the presence of an appropriate enzymatic substrate. Chloramphenical acetyl transferase (CAT) is an enzyme that metabolizes chloramphenicol and results of this reaction can be visualized by a radiometic TLC assay.

An "intracellular receptor" refers to a polypeptide or protein residing within a cell that binds a molecule, including extracellular signaling molecules, such as ligands, and initiates a response in the cell. Examples of intracellular receptors include the Ah receptor or PXR.

A "hormone receptor" refers to steroid hormone receptors that bind to hormones that diffuse into the cell across the plasma membrane. Steroid receptors such as the receptor for

thyroid hormone or vitamin D bind their ligand and then bind to specific DNA sequences within the genes that the ligand regulates. Examples include the estrogen receptor, the progesterone receptor or cortisol receptor.

A "transporter" refers to proteins within the plasma membrane that carry or otherwise direct molecules across a cell membrane. Transporters can be specific transporters for specific ligands, general transporters for a group of ligands, active transporters that utilize energy such as ATP or the electron motive force, or passive transporters that do not utilize energy of the cell. Molecules can be transported into or out of a cell depending on the transporter and the conditions that it is under. Examples include the sodium-potassium ATPases and P-glycoprotein (MDR1) that transports drug metabolites from inside the cell to outside the cell.

A "transcription factor" refers to any polypeptide or protein that can initiate or regulate transcription in a cell, such as but not limited to a eukaryotic cell. These include gene regulatory proteins that bind to enhancers and the general transcription factors that do not act in such a specific manner. Examples of transcription factors include TFIID, a general transcription factor, or a specific receptor such as PXR. HNF1 is another transcription factor that regulates expression of genes in a tissue specific manner.

To be "bound" in the sense of a polypeptide such as an, intracellular receptor, transporter or transcription factor being bound with a compound, refers to these elements being in contact such that if the polypeptide and compound are bound, then the activity of the resulting complex is different from the activity of the individual elements.

To "operably bind" is to have one element bound to another element, wherein the resulting complex can perform a function. For example, a polypeptide can bind a compound and the resulting complex can operably bind with a control sequence to modulate expression of a gene operably linked to such control sequence.

A "compound" refers to any chemical, test chemical, drug, new chemical entity (NCE) or other moiety. For example, a compound can be any foreign chemical (xenobiotic) not normally present in a subject such as mammals including humans. A compound can also be an endogenous chemical that is normally present and synthesized in biological systems, such as mammals including humans. In one aspect, oxidation of compounds by enzymes generally

results in a more water-soluble, easily excretable product. Examples include food additives, steroid hormones and drugs.

To "induce" refers to an increase in expression of a polypeptide such as an enzyme, such as enzymes involved in drug metabolism, in the presence of a compound relative to the amount of expression of such polypeptide in the absence of the compound. For example, a compound, such as a test compound, such as a drug, can induce the expression of a P450 enzyme, such that the amount of P450 enzyme produced in the presence of the compound is greater than the amount of P450 enzyme produced in the absence of the compound.

A "P450" refers to a member of a super-family of heme containing monooxygenases involved in the catalytic oxidation of xenobiotics such as drugs and endobiotics including steroid hormones. Examples include but are not limited to CYP2C9, CYP3A4 and CYP1A2.

A "glucuronyl transferase" or "UGTs" refers to polypeptides and proteins involved in glucuronidation, a major pathway that enhances the elimination of many lipophilic xenobiotics and endobiotics to more water-soluble compounds. The UDP-glucuronosyltransferase family catalyzes the glucuronidation of the glycosyl group of a nucleotide sugar to an acceptor compound at a nucleophilic functional group of oxygen, nitrogen, sulphur, and carbon with the formation of a beta-D-glucuronide product. There are over thirty five known different UGT gene products that have been divided into two subfamilies, UGT1 and UGT2, based on sequence identities. Examples include UGT1A2, UGT2B7 and UGT1A8.

A "glutathione transferase" refers to enzymes that are soluble proteins predominantly found in the cytosol of hepatocytes. These enzymes catalyze the conjugation of a variety of compounds with the endogenous tripeptide, glutathione. Cytosolic glutathione S-transferases can be divided into four families, termed alpha, mu, pi, and theta, each having different but sometimes overlapping substrate specificities. There are also microsomal glutathione transferases residing, for example, in the endoplasmic reticulum (ER). Examples include but are not limited to GST(mu) and GST(alpha).

A "sulfo transferase" refers to polypeptides or proteins such as enzymes that catalyze the sulfation of structurally diverse xenobiotics including drugs and endogenous compounds. These reactions involve the transfer of a sulfuryl group from 3'-phophoadenosine 5'-phophosulfate

(PAPS) to the hydroxyl/amino groups of acceptor molecules forming sulfuric acid esters and sulfamates. Sulfate conjugation generally results in a detoxification producing water soluble metabolites. Sulfation is also an important factor in the regulation of steroid biosynthesis and inactivation and excretion of endogenous hormones. Several isoforms of these enzymes are present in humans. Examples include but are not limited to hHST, hP-PST and hM-PST.

An "N-acetyltransferase" refers to proteins or polypeptides such as enzymes that conjugate arylamines with an acetyl group. There are distinct genes within this family of enzymes. For example, NAT1 and NAT2 that encode for N-acetyltransferase activities in humans. NAT1 activity is monomorphically distributed in human tissues, whereas NAT2 exhibits a polymorphism that allows the detection of phenotypically slow and rapid acetylators. N-acetylation of arylamines represents a competing pathway for N-oxidation, a metabolic activation step occurring in the liver. Heterocyclic amines are activated by acetylation by the NAT2 transferases.

A "P-glycoprotein" or "Pgp" refers to a product of the MDR1 gene. Its function is to transport drugs and steroids across a cell membrane. Pgp may be a determinant of the magnitude of CYP3A induction. Pgp may influence PXR ligand interaction and the CYP3A inductive response to steroids and xenobiotics.

An "enzyme" refers to a polypeptide having a catalytic activity. Detectable enzymes are enzymes that when acting upon an appropriate substrate will produce a detectable product. The detectable product is preferably detected optically, such as via the emission of light, such as fluorescence, luminescence or chemiluminescence, or by color, such as by the formation of a chromogen. Preferred detectable enzymes include, but are not limited to beta-lactamase, luciferase and beta-galactosidase.

A "detectable protein" is a polypeptide that has a physical property that is detectable. Preferred detectable proteins are proteins that are inherently fluorescent, such as Green Fluorescent Protein (GFP), SPAP renillin fluorescent protein and their derivatives.

An "extra chromosomal element" refers to a nucleic acid molecule that when present within a cell is non integrated within the genome of such cell. Examples of extra chromosomal elements include plasmids and Yeast Artificial Chromosomes (YACs).

"Within the chromosome" of a cell in the context of a nucleic acid molecule refers to a nucleic acid molecule is within the chromosome or genome of the cell as opposed to being an extra chromosomal element. A nucleic acid molecule within the chromosome of a cell can be "inserted" within the genome, such as by homologous recombination or other methods, or can be "endogenous to the chromosome." In the case of endogenous to the chromosome, the nucleic acid molecule is within the chromosome at its original locus.

To "directly" produce an event or form a structure is to not have intermediary steps or structures. For example, A and B forming AB directly interact because there is no structure between A and B. Also, C and D reacting to form E directly interact to form E because there are no intermediary steps between the reaction of C and D to form E.

To "indirectly " produce an event or form a structure is to have an intermediate step or structure. For example, A and B forming ABC have A and B indirectly interacting because there is a structure between A and B. Also, E and F forming G which reacts with H to form I is an indirect formation of I from E and F because an intermediary step is involved in the process of making I.

A "cell" is any cell, such as a prokaryotic or eukaryotic cell. A cell is preferably a eukaryotic cell and is preferably from a multi-cellular organism, but can be a unicellular organism such as a yeast or other free-living eukaryotics. A cell can be obtained from an organism, such as an animal or a human, and provided in primary culture or continuous cultures such as in the case of a cell line. A cell can be part of a population of cells, such as a population of similar cells, such as cells from the same tissue or organ, or of substantially the same cells, such as in a clonal population of cells. The cells can be obtained from any appropriate organism, such as through routine sampling, such as through biopsy for the collection of tissues or through the collection fluids, such as blood, using routine methods. Cells are preferably mammalian cells and are preferably human cells, but that need not be the case. Cells are also preferably derived from a tissue that naturally exhibit relatively high levels of expression of enzymes that are involved in drug metabolism, such as, but not limited to, liver, intestine, lung or kidney. Cells can also be transformed cells, which are cells that have been genetically altered by genetic

engineering processes, such as by the introduction of extra chromosomal elements or integration of nucleic acid molecules into the chromosome of the cell.

"High throughput screening" refers to methods for screening for activity of compounds, such as test compounds such as drugs, takes place at a rate of between about 5 assays or samples per day and about 10,000 assays or samples per day, preferably between about 10 assays or samples per day and about 1,000 assays or samples per day and more preferably between about 15 and about 500 assays or samples per day.

Introduction

The present invention provides improved cells and methods for identifying compounds that alter protein expression, such as chemicals or drugs. The invention provides other benefits as well.

As a non-limiting introduction to the breadth of the present invention, the present invention includes several general and useful aspects, including:

1) a cell that includes a first nucleic acid molecule that includes: a promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism and a reporter gene; and a second nucleic acid encoding an intracellular receptor or transcription factor; so that when the intracellular receptor or transcription factor is in contact with a compound, or directly or indirectly activated by a compound or directly or indirectly modulated by a compound, the intracellular receptor or transcription factor can operably bind with the promoter or enhancer resulting in the expression of said reporter gene; and
2) a method for evaluating compounds for the property of inducing the expression of a gene encoding a protein involved in drug metabolism, including; providing a test compound; contacting the test compound with a cell of the present invention; and detecting the expression of said reporter gene.

These aspects of the invention, as well as others described herein, can be achieved using the methods, articles of manufacture, and compositions of the present invention. To gain a full

appreciation of the scope of the present invention, it will be further recognized that various aspects of the present invention can be combined to make desirable embodiments of the invention.

1. A Cell For Evaluating Enhanced Protein Expression by Test Compounds

The present invention includes a cell that includes a first nucleic acid molecule that includes: a promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism and a reporter gene; and a second nucleic acid encoding an intracellular receptor or transcription factor; so that when the intracellular receptor or transcription factor is bound with a compound, the intracellular receptor or transcription factor can operably bind with the promoter or enhancer resulting in the expression of the reporter gene.

FIRST NUCLEIC ACID MOLECULE

The cell includes a first nucleic acid molecule that includes a promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism, including enzymes and transporters, and a reporter gene. The promoter or enhancer is operably linked to the reporter gene. In this way, when the promoter or enhancer is activated (such as by binding of a receptor/compound complex), the reporter gene is expressed. If the reporter gene is expressed at or above a detectable level, then the activation of the promoter or enhancer is reported.

The first nucleic acid molecule is preferably double stranded DNA, but that need not be the case. The first nucleic acid molecule can be extra chromosomal or be within the chromosome of the cell. Extra chromosomal elements include, but are not limited to, vectors, viruses, plasmids, YACs and linear nucleic acid molecules. Methods for preparing such plasmids, YACs and linear nucleic acid molecules that have the characteristics of the first nucleic acid molecule, such as the promoter or enhancer operably linked to the reporter gene, are known in the art. For example, nucleic acid molecules that encode promoters or enhancers operable for a nucleic acid molecule encoding a protein involved in drug metabolism are known in the art, are often times commercially available and can be prepared and cloned using routine methodologies

including PCR, restriction enzymes, digestion and chemical synthesis. These promoters or enhancers can be operably linked to a reporter gene using routine methods such that when the promoter or enhancer is activated, the reporter gene is expressed. This construct can then be cloned into an appropriate vector, such as but not limited to plasmids, viral vectors, YACs and linear nucleic acid molecules. These vectors can then be used to transform a cell or population of cells. Such transformations are known in the art, such as electroporation, viral infectivity, microballistics or passive uptake of nucleic acid molecules by cells.

If the first nucleic acid molecule includes a gene that encodes a selectable marker operably linked to a promoter, such as a constitutive promoter such as CMV promoter, MMTV promoter or SV40 promoter, cells that have taken up the nucleic acid molecule and the nucleic acid molecule is operable can be selected for. Preferred selectable markers include antibiotic resistance, such that cells that have an operable first nucleic acid molecule would be resistant to a particular antibiotic whereas cells that do not have such a first nucleic acid molecule would be susceptible to such antibiotic. In that way, cells having a first nucleic acid molecule that expresses the selectable marker can be selected and enriched. Alternative selectable markers include fluorescent proteins, such as Green Fluorescent Protein (GFP) or its derivatives, or enzymes that catalyze the formation or transformation of fluorescent substrates or products, such as beta-lactamase. Under these conditions, fluorescence activated cell sorting (FACS) can be used to isolate cells having a desired fluorescent property.

The first nucleic acid molecule can be extra chromosomal, or can be integrated within the genome of the cell. When the first nucleic acid is integrated within the genome of the cell, the first nucleic acid becomes stably integrated, which results in a cell having greater reliability and reproducibility than transiently transfected cells. Certain vectors, such as viral vectors, particularly retroviral vectors, can integrate within the genome. Also, homologous recombination can be used to promote the insertion of a nucleic acid molecule within the genome of a cell using methods, such as those described in US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000. In the alternative, transformed nucleic acid molecules can spontaneously integrate into a host genome. The integration of a first nucleic acid molecule within the genome of a cell can be

monitored by screening cells for the loss of a selectable marker or reporter gene because transiently transfected cell lines tend to eject nucleic acid molecules that are not integrated into the genome of the cell. Thus, the selectable marker or reporter gene would tend to be lost over time, such as through repeated passages of cell lines.

In one aspect of the present invention, the reporter gene is endogenous to the chromosome of the cell. In this instance, the reporter gene preferably encodes an enzyme that can readily be determined, such as by detectable enzymatic substrates or products thereof. In this instance, a nucleic acid molecule that includes a promoter or enhancer operable for the desired reporter gene is engineered into a vector such that the integration of that vector is directed to a locus in the genome at or near the reporter gene. Integration of the nucleic acid construct that includes the promoter or enhancer can be directed using homologous recombination methodologies as they are known in the art, such as those described in US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000.. Also, spontaneous or non-directed recombination methodologies can be used as they are known in the art. Not all such homologous recombination events will result in an operable link between the promoter or enhancer and the reporter gene, thus the cell or population of cells should be screened for such operable link. For example, if the event does result in an operable link, activation of the enhancer or promoter would result in the expression of the reporter gene. Such expression can be monitored and screened using appropriate detectable enzymatic substrates and/or products.

In another aspect of the present invention, the enhancer or promoter is endogenous to the chromosome of the cell. In this instance, a nucleic acid molecule that includes a reporter gene operable for the enhancer or promoter is engineered into a vector such that the integration of that vector is directed to a locus of the genome at or near the promoter or enhancer. Integration of the nucleic acid construct that includes the reporter gene can be directed using homologous recombination methodologies as they are known in the art, such as those described in US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000. Not all such homologous recombination events will result in an operable link between the promoter or enhancer and the reporter gene, thus the cell or population

of cells should be screened for such operable link. For example, if the event does restult in an operable link, activation of the enhancer or promoter would result in the expression of the reporter gene. Such expression can be monitored and screened using appropriate detectable enzymatic substrates and/or products.

SECOND NUCLEIC ACID MOLECULE

The cell also includes a second nucleic acid encoding an intracellular receptor or transcription factor. When the intracellular receptor or transcription factor is bound with a compound, the intracellular receptor or transcription factor can operably bind with the promoter or enhancer resulting in the expression of the reporter gene.

The second nucleic acid molecule is preferably double stranded DNA, but that need not be the case. The second nucleic acid molecule can be extra chromosomal or be within the chromosome of the cell. Extra chromosomal elements include, but are not limited to, vectors, viruses, plasmids, YACs and linear nucleic acid molecules. Methods for preparing such plasmids, YACs and linear nucleic acid molecules that have the characteristics of the second nucleic acid molecule, such as including a nucleic acid molecule encoding an intracellular receptor or transcription factor, are known in the art. For example, nucleic acid molecules that encode an intracellular receptor or transcription factor are known in the art, are often times commercially available and can be cloned using routine methodologies. This construct can then be cloned into an appropriate vector, such as but not limited to plasmids, viral vectors, YACs and linear nucleic acid molecules. These vectors can then be used to transform a cell or population of cells. Such transformations are known in the art, such as electroporation, viral infectivity, microballistics or passive uptake of nucleic acid molecules by cells.

Preferably, the second nucleic acid molecule includes a regulatory element, such as a promoter or enhancer, operably linked with said nucleic acid molecule encoding an intracellular receptor or transcription factor. The regulatory element is preferably a promoter or constitutive promoter, such as SV40 promoter, MMTV promoter or CMV promoter. As discussed for the first nucleic acid molecule, there are art recognized methods to make constructs such as vectors having this type of configuration.

If the second nucleic acid molecule includes a gene that encodes a selectable marker operably linked to a promoter, such as a constitutive promoter such as CMV promoter, MMTV promoter or SV40 promoter, cells that have taken up the nucleic acid molecule can be selected for. Preferred selectable markers include antibiotic resistance, such that cells that have an operable second nucleic acid molecule would be resistant to a particular antibiotic whereas cells that do not have such a second nucleic acid molecule would be susceptible to such antibiotic. In that way, cells having a second nucleic acid molecule that expresses the selectable marker can be selected and enriched. Alternative selectable markers include reporter proteins encoded by reporter genes such as fluorescent proteins, such as Green Fluorescent Protein (GFP) or its derivatives, or enzymes that catalyze the formation or transformation of fluorescent substrates or products, such as beta-lactamase. Under these conditions, fluorescence activated cell sorting (FACS) can be used to isolate cells having a desired fluorescent property.

In aspects of the invention where the first nucleic acid molecule and the second nucleic acid molecule both include selectable markers, it is preferable that these selectable markers be different, but that need not be the case. Different selectable markers allows the independent monitoring of both the first nucleic acid molecule and the second nucleic acid molecule in the cell.

The second nucleic acid molecule can be extra chromosomal, or can be integrated within the genome of the cell. When the second nucleic acid is integrated within the genome of the cell, the second nucleic acid becomes stably integrated, which results in a cell having greater reliability and reproducibility than transiently trasfected cells. Certain vectors, such as viral vectors, particularly retroviral vectors, can integrate within the genome. Also, homologous recombination can be used to promote the insertion of a nucleic acid molecule within the genome of a cell using methods such as those described in US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000. In the alternative, transformed nucleic acid molecules can spontaneously integrate into a host genome. The integration of a second nucleic acid molecule within the genome of a cell can be monitored by screening cells for the loss of a selectable marker or reporter gene because transiently trasfected cell lines tend to eject nucleic acid molecules that are not integrated into the genome of

the cell. Thus, the selectable marker or reporter gene would tend to be lost over time. Materials and methods for integrating nucleic acid molecules within the chromosome are known in the art (see, for example, WO 98/13353, published April 2, 1998, naming Whitney *et al.* as inventors; WO 94/24301, published October 27, 1994 to The University of Edinburgh; US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000.).

In one preferred aspect of the present invention, the gene encoding the intracellular receptor or transcription factor is endogenous to the chromosome of the cell. In particular, the gene encoding the intracellular receptor or transcription factor is in its native environment within the cell's genome, that is to say that its location and surrounding genome that includes cis-acting regulatory elements such as promoters or enhancers has not been purposely altered by human intervention.

In another aspect of the present invention, the gene encoding an intracellular receptor or transcription factor is endogenous to the chromosome of the cell but an exogenous regulatory sequence operable for the gene encoding an intracellular receptor or transcription factor, such as a promoter or enhancer, is integrated into the genome of the cell, preferably as to be operably linked with the endogenous gene encoding an intracellular receptor or transcription factor. Integration of the nucleic acid construct that includes the gene encoding an intracellular receptor or transcription factor can be directed using homologous recombination methodologies or spontaneous non-directed recombination methods as they are known in the art, such as those described in US Patent No. 6,187,305 to Treco *et al.*, issued February 13, 2001 and U.S. Patent No. 6,063,630 to Treco *et al.*, issued May 16, 2000..

Not all such homologous recombination events will result in an operable link between the regulatory element and the gene encoding an intracellular reporter or transcription factor, thus the cell or population of cells should be screened for such operable link. Such expression can be monitored and screened using methods appropriate for detecting the activity of such intracellular receptor or transcription factor. In the alternative, the construct to be integrated within the genome of the cell can include a reporter gene that is operably linked with the regulatory sequence used to modulate the expression of the intracellular receptor or transcription factor. In

the alternative, a the reporter gene can be operably linked with a second regulatory sequence, which can be the same or different from the regulatory sequence used to modulate the expression of the intracellular receptor or transcription factor. In this instance, the sustained expression of the reporter gene indicates that the nucleic acid construct had operably integrated into the genome of the cell.

INTERACTION OF FIRST NUCLEIC ACID MOLECULE AND SECOND NUCLEIC ACID MOLECULE

FIG. 1 through **FIG. 5** depict various aspect of the present invention in diagrammatic sketches. These sketches provide the general workings of the present invention under circumstances where the first nucleic acid molecule and the second nucleic acid molecule are exogenous, endogenous, integrated or extra chromosomal. **FIG. 1** depicts the general interactions of the first nucleic acid molecule and the second nucleic acid molecule. In general, when the cell is contacted with a compound that induces the expression of the enzyme or transporter involved in drug metabolism, the reporter gene is expressed. However, if the cell does not have the genes encoding the enzyme involved in drug metabolism or if such genes are not in a configuration that allows expression, the protein involved in drug metabolism may not be expressed.

As depicted in **FIG. 1A**, the second nucleic acid molecule (**10**) within a cell (**16**) includes a regulatory element (**12**) to modulate the expression of a gene encoding an intracellular receptor or transcription factor (**14**). The expressed intracellular receptor or transcription factor (**18**) can then interact with a test compound (**11**) by appropriate interactions, such as binding, associating, modulating and the like. The test compound can enter the cell by way of active transport or passive transport mechanisms. The test compound may optionally be modified by this transport process to form a modified test compound (**13**). As shown in **FIG. 1B**, the transcription factor or receptor can specifically bind with an appropriate test compound or metabolite if they are receptor - ligand pairs to form a complex (**17**). This complex (**17**) can bind with the first nucleic acid molecule (**19**) and optionally with the genome of the cell (**20**). When binding with the first nucleic acid molecule (**19**) or the genome of the cell (**20**), the complex (**17**) can bind with the

regulatory element operable for a nucleic acid molecule encoding a protein involved in drug metabolism (**22**) or with an endogenous regulatory element (**24**) that can bind with such complex (**17**). In the latter case, the endogenous regulatory element can modulate the expression of a gene encoding a protein involved in drug metabolism (**26**). However, binding to the endogenous regulatory element is not a requirement of the present invention, particularly in this aspect of the present invention. As shown in **FIG. 1C**, the binding of the complex to the regulatory element on the first nucleic acid molecule results in the expression of a reporter (**28**) encoded by a reporter gene (**30**). The binding of the complex to the endogenous regulatory sequence can result in the expression of an endogenous protein involved in drug metabolism (**21**). The endogenous protein involved in drug metabolism can modify a compound (**23**) via a variety of mechanisms, such as by hydroxylation (**25**). The reporter can be detectable, such as by fluorescence of the reporter (**27**) or by the conversion of a substrate (**29**) to a detectable product (**31**) (**FIG. 1D**).

Thus, when the compound (**11**) or a modified compound (**13**) capable of binding with the intracellular receptor or transcription factor (**18**) and binding with a regulatory sequence for a nucleic acid molecule encoding a protein involved in drug metabolism (**22**), the reporter gene (**30**) is expressed as a reporter (**28**) which can be detected.

PROTEINS INVOLVED IN DRUG METABOLISM

The protein involved in drug metabolism can be any appropriate enzyme or transporter. Preferred enzymes involved in drug metabolism include but are not limited to P450s, transporters, glucuronoyl transferases, N-acetyl transferases, glutathione transferases, p-glycoproteins and sulfo transferases. Preferred transporters include but are not limited to p-glycoprotein (MDR1). This protein transports drug metabolites out of a cell and can influence the rate of drug metabolism by a cell. P-glycoprotein expression may be altered by certain drugs (see, for example, Schuetz *et al.* (1999) *Mol. Pharmacol.*, 49:311-318; Lan *et al.* (2000) *Mol. Pharmacol.*, 58:863-869, and Wrighton *et al.* (2000) *Drug Metab. Rev.*, 32:339-361). Nucleic acid molecules encoding these types of proteins have been reported and can be isolated using standard methods in molecular biology (see, for example, Garattini (1997) *Drug Metab. Rev.*,

29:853-886; Schuetz *et al.* (1996) *Mol. Pharmacol.*, 49:311-318) and Nebert and Dieter (2000) *Pharmacology,* 61:124-135).

PROMOTER OR ENHANCER

The regulatory sequences, such as promoters or enhancers, operable for a nucleic acid molecule encoding a protein involved in drug metabolism is preferably a promoter or enhancer for P450s, glucuronyl transferases, glutathione transferases and sulfo transferases or p-glycoprotein. Sequences of such regulatory sequences are known in the art and can be isolated using standard methods in molecular biology (see, for example, Nelson *et al.* (1993) *DNA Cell Biol.*, 12:1-51; Windmill *et al.* (1997) *Mutat. Res.*, 376:153-160; Schuetz *et al.* (1995) *J. Cell Physiol.*, 165:261-272; Schuetz *et al.* (1996) *Mol. Pharmacol.*, 49:311-318; Parker *et al.* (1995) *J. Clin. Endocrin. Metabol.*, 80:1027-1031; Brockmöller *et al.* (1998) *Toxicol. Lett.*, 103:173-183; Vaury *et al.* (1995) *Cancer Res.*, 55:5520-5523; Rodrigo *et al.* (1999) *Scand. J. Gastroenterol.*, 34:303-307; and Munzel *et al.* (1999) *Drug Metab. Dispos.*, 27:569-573).

REPORTER GENE

The reporter gene can be any appropriate reporter gene as is known in the art. A reporter gene encodes a reporter, such as a detectable protein or a detectable enzyme. Detectable proteins can be detected based on their physical characteristics, such as fluorescence in the case of fluorescent proteins such as Green Fluorescent Protein (GFP) or its derivatives. Enzymes can be detected using appropriate substrates that change properties when a protein acts on the substrate to form a product. Certain substrate - enzyme pairs can cause a change in fluorescent properties of the substrate, such as in the case of beta-lactamase acting on CCF2/AM to alter the characteristics of FRET in the CCF2/AM molecule. Fluorescence can be generated in the pair of glucuronidase activity on MUG. Chemiluminescence can be generated by activity of luminol dioxanes. Luminescence can be generated by luciferase activity on luciferin (see, for example, Alam and Cook (1990) *Anal. Biochem.*, 188:45-254). Colored product can be generated by beta-

galactosidase activity on X-Gal substrate. The applicability of reporter genes to the study of reporter gene transcription has been discussed (Alam and Cook (1990) *Anal. Biochem.*, 188:45-254).

INTRACELLULAR RECEPTOR OR TRANSCRIPTION FACTOR

In one aspect of the present invention, the intracellular receptor or transcription factor forms a complex with a xenobiotic such as a drug, chemical or metabolite thereof and directly or indirectly produces transcriptional activation of a gene encoding a protein involved in drug metabolism. This activity is depicted in the figures. In one aspect of the present invention, the intracellular receptor or transcription factor is not a hormone receptor, but that is not a requirement of the present invention. In another aspect of the present invention, the intracellular receptor or transcription factor is an orphan receptor, that is, a receptor that does not have a known or identified function. Examples of such orphan receptors include, but are not limited to, PXR and CAR (see, for example, Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023; Jones *et al.* (2000) *Mol. Endocrinol.*, 14:27-39; Honkakoski *et al.* (2000) *Biochem. J.*, 347:321-337; and Savas *et al.* (1999) *Mol. Pharmacol.*, 56:851-857). The intracellular receptor or transcription factor can be a hormone receptor, such as but not limited to the glucocorticoid receptor.

CELLS

The cells of the present invention can be any cell, including prokaryotic or eukaryotic. Cells are preferably eukaryotic and are from a mammalian subject, including a human. The cells can be of any origin, such as derived from the mesoderm, endoderm or ectoderm. The cells can be derived from any tissue, organ or fluid from a subject, but are preferably derived from the liver, kidney or lung. The cells can be provided from a subject, such as from a sample from a biopsy or autopsy, and can be primary cells such are known or can be made using methods known in the art. The cells can also be a cell line, such as are known or can be made using methods known in the art. For example, a variety of cell lines are available from the American Type Tissue Collection (see, ATCC Catalogues (2001)). The cells can also be a mixed culture such as a variety of cells or cell types are provided. For example, primary cells can include a

variety of cell types, such as hepatocytes mixed with fibroblasts. Mixed cultures of different continuous cell lines or mixed cultures of primary cells and continuous cell lines can also be used. In one aspect of the present invention, the cells can be transformed such that they can express an exogenous protein or polypeptide.

In one aspect of the present invention cells can be provided from a particular subject. The identity of the subject need not be known, only that a particular subject is the source of cells. In the alternative, cells from a population of subjects, such as those having common ethnic origin or common disease states, disease conditions, physiological genotypes or phenotypes or metabolic phenotypes or genotypes can be used. These cells can be transformed to become cells of the present invention and can be used in the methods of the present invention. In this instance, the response of these cells to xenobiotics can be indicative of how that subject or population of subjects would respond metabolically and physiologically to that xenobiotic. In the case of cells from a population of subjects, cells from different subjects can be tested separately, but that need not be the case. The results of these types of studies can be collected and analyzed using bioinformatic technologies to assist in pharmacogenomic studies and methods. A variety of computer programs are available to provide such analyses, such as but not limited to statistical software that can provide linear or non-linear statistical methodologies. The selection of statistical analysis can be chosen by the skilled artisan.

The data, analysis and/or results generated using these methods is also part of the present invention. The data, analysis and/or results can be stored on appropriate information storage media, such as but not limited to magnetic media, tapes, paper or the like. The information storage media is preferably in a machine readable format, but that need not be the case. The information storage media can also be part of a machine, such as a machine having a central processing unit. Such a machine can be operating or not operating to be part of the present invention.

II. A METHOD FOR EVALUATING A TEST COMPOUND FOR INDUCING EXPRESSION OF A GENE ENCODING A PROTEIN INVOLVED IN DRUG METABOLISM

The present invention includes a method for evaluating compounds for the property of inducing the expression of a gene encoding a protein involved in drug metabolism, including: providing a test compound, contacting the test compound with a cell of the present invention and detecting the expression of said reporter gene. The expression of the reporter gene is indicative that the test compound altered the expression of a gene encoding a protein involved in drug metabolism. The method can be in a high throughput method, but that is not a requirement of the present invention.

Various aspects of the present invention are depicted in the figures. For example, **FIG. 1** depicts a series of figures for one aspect of the present invention, where the first nucleic acid molecule and second nucleic acid molecule are provided as extra chromosomal elements such as plasmids. As depicted in **FIG. 1A**, a regulatory element P2 modulates the transcription of the gene encoding an intracellular receptor or transcription factor. The translation product can then interact with a test compound that binds with the intracellular receptor or transcription factor. As depicted in **FIG. 1B**, the complex of the intracellular receptor or transcription factor and xenobiotic or test compound can then bind with the promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism. The complex can also enter the nucleus and optionally bind with the endogenous promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism, if present or active in such cell. Upon binding of this complex with the promoter or enhancer operable for a nucleic acid molecule encoding a protein involved in drug metabolism, a reporter gene is transcribed and translated into a reporter and optionally the endogenous enzyme involved in drug metabolism is expressed, if present or active in such cell (**FIG. 1C**). That reporter can be detectable by its physical properties, such as fluorescence, or can be a protein that is detectable based on its enzymatic conversion of substrate to product, such as a detectable product (**FIG. 1D**). In another

aspect of the present invention, both the first nucleic acid molecule and the second nucleic acid molecule are provided on the same extra chromosomal element, such as a single plasmid or YAC or separate plasmids.

Alternatives to the aspect of the present invention depicted in **FIG. 1** are also provided. For example, **FIG. 2** depicts the case where the first nucleic acid molecule is an extra chromosomal element whereas the second nucleic acid molecule is endogenous to the chromosome of the cell. **FIG. 3** depicts the case where the first nucleic acid molecule is an extra chromosomal element and the second nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell. **FIG. 4** depicts the case where the first nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell and the second nucleic acid molecule is endogenous to the chromosome of the cell. **FIG. 5** depicts the case where the first nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell and the second nucleic acid molecule is an exogenous nucleic acid molecule integrated into the genome of the cell.

The methods of the present invention can be carried out using appropriate hardware, such as tissue culture flasks or plates having appropriate surface area per flask or well. Preferably, the methods utilize appropriate plates having six, twelve, twenty-four, fourty-eight or ninety-six wells on a standard size microtiter plate footprint. The methods can also utilize plates having higher well densities, such as 192, 288, 384, 480, 576, 672, 768, 864, 960, 1056 or higher wells per plate on a standard footprint. These plates are commercially available such as through Costar and other vendors in the commercial marketplace.

The methods can be performed using human technical resources, or in part or in whole using robotics. In the later case, robotics can be used to provide high throughput capabilities that can reduce the cost and increase the reliability of the performance of the methods. Robotic systems can be made to perform these methods. For example, sample storage units known in the art can be used to store test compounds in an indexed fashion. Retrieving robotics known in the art can be used to retrieve samples from the sample storage unit for later dispensation into test vessels, such as wells of a microtiter plate, using dispensation robotics known in the art.

Robotics can be used to dispense cells of the present invention and appropriate culture materials into test vessels using dispensation robotics known in the art, which can then be cultured under appropriate conditions to grow or maintain such cell cultures. Incubators, such as those known in the art, can be used to provide appropriate conditions.

Cell cultures in test vessels can be combined with test compounds using robotics, such as using dispensation robotics known in the art. The cells with test compounds can be provided appropriate conditions, such as atmosphere and temperature, for a method of the present invention, such as in an incubator as is known in the art. Reporter gene products can be detected directly, such as with detectable proteins, or with the addition of enzymatic substrates for enzymes. Enzymatic substrates can be added to test vessels using robotics, such as dispensation units. Cells can be lysed, if needed, desired or appropriate using appropriate reagents, which can be dispensed using robotic dispensation devices and methods known in the art. Detection devices known in the art, such as microtiter plate readers for chromogens, fluorescence, luminescence or the like, can be used to detect reporter gene products.

The information output or data generated using these methods can be routed to information storage devices, such as devices that include a central processing unit. The information storage device can also include information processing capabilities, such as appropriate software. This software can have the capability of making statistical comparisons or performing statistical analysis such as is known in the art, including linear and non-linear methodologies.

Such robotic systems and their components are generally known in the art and are generally described or commercially available in whole or in part from a variety of commercial vendors (see, generally WO 98/52047, published November 19, 1998, naming Stylli *et al.* as inventors). The various steps and processes used to perform a method of the present invention can independently be performed by robotics or humans.

EXAMPLES

EXAMPLE I

1. MATERIALS AND METHODS

Construction of Plasmids for Transfections

The full length coding region of human PXR was derived by RT-PCR from RNA obtained from a human liver sample. The forward and reverse oligonucleotide sequences were 5'-ATGGAGGTGAGACCCAAAGAA-3' (**SEQ ID NO. 1**) and 5'-CTCAGCTACCTGTGATGCCGA-3' (**SEQ ID NO. 2**), respectively. The PCR conditions consisted of denaturing at 94°C for four minutes, followed by thirty cycles of 94°C for 45 seconds, 55°C for one minute and 72°C for two minutes with a final extension at 72°C for seven minutes. The 1300 base pair amplified product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and subjected to sequence analysis. The sequences obtained agreed over the entire coding region with that previously described (Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023). The cDNA was then extracted from pCR2.1 by digestion with BamH1 and Not1 and cloned into analogous sites of a pIRES(neo) vector (Clontech, Palo Alto, CA) containing a neomycin selection cassette.

Forward and reverse primers were made to a 5'-flanking region of CYP3A4, known to contain the PXRE (Quattrochi *et al.* (1995) *J. Biol. Chem.*, 270:28917-28923). The forward and reverse oligonucleotide sequences were 5'-AGACTCACCTCTGTTCAGGGAAA-3' (**SEQ ID NO. 3**) and 5'-CACCTTGGAAGTTGGC-3' (**SEQ ID NO. 4**) respectively. This 480 base pair region was amplified by PCR from genomic DNA isolated from a sample of human liver. The amplimer was cloned into pCR2.1 and sequenced. The enhancer region was then liberated from pCR2.1 with EcoR1, blunt-ended and subsequently cloned into the Sma1 site of the pGL3-promoter vector (Promega, Madison, WI) without a mammalian selectable marker and including

a luciferase reporter gene. Sequence analysis verified that the enhancer was identical to that previously published (Quattrochi *et al.* (1995) *J. Biol. Chem.*, 270:28917-28923) and that the oligonucleotide had been inserted.

Stable Transfections and Selections of G418-Resistant Colonies

HepG2 cells were harvested at approximately 50% confluency and seeded in six well dishes at 5 x 10^5 cells per well in DMEM containing 10% fetal bovine serum (FBS). After twenty four hours recovery, cells were transfected with the following combinations: CYP3A4 enhancer/pGL3promoter and hPXR/pIRES(neo) at a ratio of 5:1 (six micrograms total DNA/well), CYP3A4 enhancer /pGL3 promoter and pIRES(neo) (5:1 ratio, six micrograms DNA per well), pGL3promoter and pIRES(neo) (5:1 ratio, six micrograms DNA/well) and pGL3promoter and hPXR/pIRES (5:1 ratio, six micrograms of DNA per well) using a modification of the calcium phosphate co-precipitation procedure (Ausubel *et al.*, Current Protocols in Molecular Biology, Green Publishing Associate/Wiley Interscience, New York (1990)). The control cells were those that received plasmid DNA containing pGL3 promoter and pIRES(neo) or pGL3promoter and hPXR in pIRES(neo). After sixteen hours of exposure to the precipitated DNA, the culture medium was removed, cells washed twice with DMEM, and fresh media containing 10% FBS added. After an additional twenty four hours, media was replaced with that containing 400 micrograms per milliliter of G418. Media was changed every two days for three weeks until small colonies were visible. Single colonies were selected and transferred to twenty four well Costar plates (VWR, Westchester, PA). Each of the twenty four wells contained the same media and cells were grown to confluency with media changes every three days. Confluent wells were trypsinized and cells transferred into six well plates and upon reaching confluency therein, cells were further transferred to T75 flasks. Confluent flasks of randomly selected colonies were trypsinized and used to seed 96 well plates to measure rifampicin-induced luciferase response of individual colonies to test for the presence of recombinants.

Luciferase Assay

Luciferase assays were performed as specified by the manufacture (LucLite system, Packard Instrument, Meriden, CT). Activity was determined using the Packard LumniCount luminometer and results expressed as relative light units or fold increase above control (DMSO treated cells).

Treatment of Stably Transformed Cells

The HepG2 derived cell lines containing recombinant DNA were grown as monolayers in media including Dulbeccos's Modified Eagle's Medium (DMEM, Gibco/BRL, Gaithersburg, MD), 50 U/ml penicillin, 100 micrograms per milliliter streptomycin, 0.1 millimolar non-essential amino acids (Gibco/BRL), 0.4 milligrams per milliliter G418 (Gibco/BRL) 10% fetal bovine serum (FBS, Hyclone. Logan, UT) and maintained in an atmosphere of 5% CO_2 and 95% air at 37°C. Cells were seeded in T75 flasks and grown to confluency. After three to five days, cells were removed from flasks by trypsinization and replated on 96 well plates at a density of about 1.0×10^4 cells per well in DMEM media containing 0.1% FBS and G418 but without indicator (phenol red). After a seventy two hour recovery, the hepatomas, control and CYP3A4 enhancer containing cells and those with hPXR + 3A4 enhancer, were treated with 0.1% DMSO (control) or inducer dissolved in DMSO for various time periods and concentrations in fresh media containing 0.1% FBS and G418 without indicator. That cells contained the CYP3A4 enhancer was verified by comparing results to control cells transfected with hPXR/pIRES(neo) and pGL3promoter or pIRES(neo) and pGL3promoter. Screening for cells containing the pGL3/3A4 enhancer were performed by treatment with ten micromolar rifampicin and 0.1% DMSO. Those cells exhibiting greater than three-fold increases in luciferase activity above control (DMSO-treated) cells were considered transformed with the correct plasmids. Finally, copy number of the 3A4 enhancer integrated into the genome of the HepG2 cells was verified by Southern blot analysis.

To test the cell lines considered positive for the pIRES(neo) and pGL3/3A4 or hPXR + pGL3/3A4, time course studies were performed. Cells were treated with ten micromolar rifampicin for six to seventy eight hours, with analysis of response determined at six hour

intervals. In addition, dose response curves were constructed for various CYP3A4 inducers and non-inducers to confirm the specificity of the response element. The dose-response curves consisted of concentrations ranging from 1 to 1,000 micromolar at five different doses. The agents tested were RU486 (Biomol, Plymouth Meeting, PA), mevastatin (Biomol), rifampicin (Sigma Chemical, St. Louis, MO), omeprazole (Astra-Zeneca, Sweden), clotrimazole (Sigma), phenobarbital (Merck, West Point, PA), or dexamethasone (Sigma) and as negative inducers, pregnenolone 16(alpha)-carbonitrile (PCN, Sigma) and TCDD (Chemsyn Science Laboratories, Lenexa, KY). Cells were exposed to each compound for 72 hours. All inducers were dissolved in DMSO (Sigma Chemical, St. Louis, MO) and this solvent was added to control cells at 0.1%.

2. RESULTS

Identification of G418-Resistant Colonies Expressing Inducible Luciferase Activity

Stable cell lines were developed by transfection of the plasmids, p3A4-enhancer, phPXR, and control vectors into HepG2 cells and selecting from G418 resistance. Resistant colonies were identified for the p3A4-enhancer, p3A4-enhancer-phPXR, and control vectors (**TABLE 1**). Southern blot analysis of total cellular DNA from several transformants confirmed the presence of stably integrated CYP3A4 enhancer sequences. Validation that hPXR was stably integrated into cells receiving this plasmid was by Northern blot analysis of several colonies (**FIG. 8**). When compared to RNA from HepG2 cells not transformed with phPXR or primary cultures of human hepatocytes, PXR mRNA was significantly over-expressed. Randomly selected colonies were tested for inducible luciferase activity by treatment with DMSO or ten micromolar rifampicin. **TABLE 1** summarizes the number of G418-resistant colonies screened for luciferase activity and the number of G418-resistant colonies having either basal or induced luciferase activity.

TABLE 1

		Number of Colonies With:	
DNA used in transfection	**Number of colonies screened for luciferase activity**	**Basal luciferase activity (a)**	**Induced luciferase activity (b)**
p3A4 enhancer	13	11	6
p3A4 enhancer and phPXR	96	79	36
Control (pluciferase plus phPXR)	6	3	0
HepG2 cells were harvested at approximately 50% confluency and seeded in 6-well dishes at about 5 x 10^5 cells per well in DMEM containing 10% FBS. After twenty four hours recovery, cells were transfected. After an additional forty hours, media was replaced with that containing 400 micrograms per milliliter G418. Media was changed every two days for three weeks until small colonies were visible. Individual G418-resistant colonies were expanded and tested for recombinants by treatment with 10 micromolar rifampicin, followed by analysis of luciferase activity.			
(a) Defined as four times background.			
(b) Defined as the ratio of rifampicin-treated to DMSO-treated.			

Transfection of the p3A4-enhancer plasmid into HepG2 cells, followed by G418 selection, resulted in the isolation of several G418-resistant colonies, of which thirteen were tested for luciferase activity. Eleven G418-resistant colonies were able to support basal-level luciferase expression and six colonies supported inducer-mediated luciferase activity when treated with ten micromolar rifampicin for forty-eight hours or seventy-two hours.

Thirty-six G418-resistant colonies containing the stably integrated p3A4-enhancer and phPXR plasmids showed high levels of luciferase expression with rifampicin treatment (**TABLE 1**). Three control G418-resistant colonies (clones) harboring the pIRES(neo) + pluciferase plasmids exhibited basal level luciferase activity. The p3A4-enhancer + phPXR and p3A4-

enhancer transformants containing the highest inducible luciferase activity were chosen for further studies and designated PXR/3A4 (colony 1F) and 3A4 (colony 13), respectively.

Inducible Luciferase Activity From Stably Integrated CYP3A4 Sequences

The initial experiments performed in 96 well plates consisted of a time response curve for the p3A4-enhancer + phPXR, p3A4-enhancer, and the vector control cells. Exposure to ten micromolar rifampicin ranged from zero to seventy two hours (**FIG. 9**). For colony 3A4/13, rifampicin-mediated induction of luciferase activity was apparent at seventy two to seventy eight hours following exposure and ranged from 35 fold to 43 fold above cells treated with DMSO. Two separate colonies containing CYP3A4 enhancer and hPXR, colonies 1F and 6H, exhibited luciferase activity that was 2.8 to 3.8 fold above DMSO treated cells upon seventy two hours of exposure to ten micro molar rifampicin (**FIG. 10**). In addition, various amounts of cells were added to each well to determine the preferred or optimal amount, for example gave the greatest response with the least background (**FIG. 11**). This number (fifty microliters) reflected the amount of cells needed to produce a readily detectable luciferase signal, low background levels, and that amount that would not alter the pH of the media over a seventy-two hour drug exposure period.

Finally, whether serum had an affect on background luciferase activity was tested using a control transformant containing the phPXR + pGL3promoter (**FIG. 12**). The results also indicate that serum did not alter luciferase activity.

Induction of CYP3A4 in Human Hepatocytes

Human hepatocytes were treated with various CYP3A4 inducers for forty eight hours, harvested, and RNA and cell homogenates isolated. **FIG. 13** depicts the results of Northern blot analysis on RNA from primary cultures treated with various inducers including dexamethasone (ten micromolar), phenobarbital (one millimolar), rifampicin (ten micromolar), clotrimazole (ten micromolar), and RU486 (ten micromolar). Results indicate that cells exposed to media without dexamethasone did not express CYP3A4. In 0.1 and 10 micromolar dexamethasone, CYP3A4 levels are apparent. Indeed, ten micromolar dexamethasone significantly increased eight-fold

CYP3A4 mRNA. Moreover, rifampicin produced a 7.8-fold increase in 3A4 message above that observed in cells exposed to 10^{-7} M dexamethasone in 0.1% DMSO. Whereas phenobarbital, clotrimazole and RU486 slightly increased CYP3A4 message 3.8-fold, 4.9-fold and 1.7-fold, respectively, over 0.1% DMSO and 10^{-7} M dexamethasone treated cells.

High Throughput System Containing Stable Cell Lines

Using the 96-well plate high throughput format, various inducers and non-inducers of CYP3A4 were examined. Each chemical was applied to the cells at different concentration in quadruplicate. Both cells containing the PXR + 3A4 enhancer and those with only the 3A4 enhancer (without exogenous hPXR, colony 13)were examined. **FIG. 14** and **FIG. 15** depict the change in luciferase activity in stably transformed cells (colony 1F) harboring both p3A4 and phPXR treated with various known CYP3A4 inducers and two non-inducers, namely TCDD and PCN at single concentrations. At single concentrations, omeprazole appeared to produce the largest response when compared to the other inducers, while PCN and TCDD produced minimal luciferase activity, less than two-fold. Colony 13 harboring the 3A4 enhancer and luciferase produced greater fold increases in luciferase activity for all inducers when compared to colony 1F. Omeprazole, clotrimazole and RU486 produced the largest induction while PCN and TCDD produced less than one-fold increase. When three different concentrations of each inducer were tested in colony 13, 100 micromolar omeprazole produced the largest induction. Rifampicin (25 micromolar) plus 10 micromolar clotrimazole also produced between 40-fold and 45-fold increase (**FIG. 16**). These results indicate that cell lines harboring the CYP3A4 enhancer are efficient at screening inducers and that the addition of hPXR in constructing the stable transformants does not increase the induction of CYP3A4.

EXAMPLE II

In this example, the effects of several agents, such as dietary flavonoids, on CYP1A1 expression utilizing a high throughput screening system for assessing human CYP induction, are examined. HepG2 cells stably integrated with regulatory regions of human CYP1A1 were treated with resveratrol, apigenin, curcumin, kaempferol, green tea extract (GTE), (-) epigallocatechin gallate (EGCG), quercetin, and naringenin. Of these flavonoids, resveratrol produced the largest increase in CYP1A1-mediated luciferase activity (ten-fold) whole GTE, apigenin, curcumin and kaempferol produced two-fold to three-fold increases in activity. In caparison to TCDD, omeprazole or benzanthracene, where increases in luciferase activity ranged from twelve to thirty-five fold, these flavonoids exhibited weak agonist activity. The remaining compounds, EGCG, quercetin, and naringenin produced negligible effects. Cotreatment of cells with TCDD and GTE, naringenin and apigenin resulted in fifty-eight, seventy-seven and seventy-four percent reductions, respectively, in TCDD-mediated CYP1A1 induction, indicating that these flavonoids exhibit potential antagonist activity towards the Ah receptor. Furthermore, results indicate that GTE and apigenin possess Ah receptor antagonist and weak agonist activities. Also disclosed is a 96-well plate assay for high throughput screening for P450 induction in less than twenty-four hours, was efficient for determining the effects of flavonoids on human CYP1A expression. Signal to noise ratios were low and well-to-well and replicate variability was below ten percent allowing induction to be easily detected in this system. These features illustrate the reliability and feasibility of this high volume screening system for identifying CYP inducers. Furthermore, results produced with the stable cell line were corroborated in HepG2 cells and primary cultures of human hepatocytes, indicating that stably integrated cell lines harboring enhancer elements of a P450 gene can be utilized in high throughput screening systems.

1. Materials and Methods

Cell Cultures and Treatment

Cell line 101L (University of California San Diego), derived from human hepatoma cell line HepG2 (ATCC, Wistar Institute), was stably transfected with the human CYP1A1 promoter and the 5' flanking sequences linked to the luciferase reporter gene (see, Postlind *et al.* (1993) *Toxicol. Appl. Pharmacol.*, 118:255-262). Briefly, the 101L cell line was established by stably transfecting a plasmid containing the human CYP1A1 promoter (-3275 to +89) linked to the firefly luciferase reporter gene into the human hepatoma cell line, HepG2. The CYP1A1 promoter region contains three DREs ad the cell line was estimated to contain two copies of the integrated plasmid.

The 101L cell line was grown as monolayers in media including Dulbecco's Modified Eagle's Medium (DMDM, Gibco/BRL), 50 U/ml penicillin, 100 micrograms/ml streptomycin, 0.1 millmolar essential amino acids (Gibco/BRL), 0.4 milligrams/ml G418 (Gibco/BRL), 10% fetal bovine serum (FBS, Hyclone, Logan UT) and maintained in an atmosphere of 5% CO_2 and 95% air at 37°C. Cells were initially seeded in flasks containing media without G418. After an overnight incubation the cultures were changed into media containing G418 for antibiotic selection. After three to five days, cells were removed from flasks by trypsinization and replated on either a twenty-four-well plate at a density of 3.5×10^5 cells per well, or a ninety-six-well plate at a density of 7.5×10^4 cells per well, in DMEM media that was replaced with that containing 0.1% FBS and without G418 or indicator (phenol red). The next day, media containing 0.1% FBS and G418 was added to the cultures. After twenty-four hours, cells containing stably integrated reporter constructs were treated with 0.1% DMSO (control), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Chemsyn Science Laboratories, Lenexa, KY), 3-methylcholanthrene (3-MC, Sigma Chemical Co., St. Louis, Mo), benzanthracene (BA, Sigma), omeprazole (Astra-Zeneca, Sweden), rifampicin (Rif, Sigma), quercetin (Sigma), green tea extract (GTE, Sigma), resveratrol (Sigma), apiginen (Sigma), curcumin (Sigma), kaempferol (Sigma), (-)-epigallocatechin gallate (EGCG, Sigma), or naringenin (Sigma) in fresh media containing 0.1% FBS and G418 without indicator. For the antagonist experiments, cells were co-

treated with a flavonoid and two nanomolar TCDD. All inducers were dissolved in DMSO and this reagent was added to control cells at 0.1%. The cells were treated with varous doses and times (six to eighteen hours). After treatment, the media containing the compound was removed by aspiration and replaced with one-hundred microliters per well of DMEM for direct analysis of luciferase activity. Experiments were performed on 101L cells from frozen stocks on the initial derivation and the passage number was limited to thirty. The latter passages exhibited responses similar to those of the earliest passage.

Luciferase Assay

Luciferase assays were performed as specified by the manufacturer (LucLite system, Packard Instrument, Meriden, CT). Activity was determined using a Packard LumiCount luminometer and results expressed as relative light units or fold increase above control (DMSO treated cells.

HepG2 Cultures and Treatment

HepG2 cells were obtained from American Type Culture Collection (ATCC). Cells were grown in DMEM (Gibco/BRL). Twenty-four hours after cells were plated and grown to confluency, they were treated with one of the bioflavonoids, TCDD, or beta-naphthoflavone (Sigma). All inducers were dissolved in DMSO and this solvent was added to control cells at 0.1%.

Primary Cultures of Human Hepatocyte and Treatment

Six well plates containing human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTPADS, University of Minnesota, Minneapolis, MN). Upon arrival, media was replaced with that containing Human Hepatocyte Maintenance Media (HHMM, Clonetics, San Diego, CA) (Runge *et al.* (2000) *Biochem. Biophys. Res. Commun.*, 273:333-341) and maintained in an atmosphere of 95% air and 5% CO_2 at 37°C. The following day, cells were treated for twenty-four hours with 0.1% DMSO (control), 50 micromolar benzanthracence, 2 nanomolar TCDD, 20 micromolar kaempferol, 20 micromolar resveratrol, or

20 micromolar naringenin, 10 micromolar apigenin, 0.1 milligrams/ml GTE or co-treated with TCDD and a flavonoid. All inducers were dissolved in DMSO and added to media at a 0.1% final concentration of this reagent. After treatment, media was removed and cells harvested for RNA isolation.

RNA Isolation and Northern Blot Analysis

Total RNA from hepatocytes or HepG2 cells was isolated using Trizol™ reagent (Gibco BRL Products, Gaithersburg, MD) and quantified by measuring absorbance at 260 nm; purity was assessed by determining the 260/280 nm ratio. Northern blot analysis was performed by electrophoresis of total RNA (10 micrograms) through a 1% agarose-2.2 M formaldehyde gel, followed by blotting onto a nylon membrane (MSI, Westboro, MA) (Shih *et al.* (1999) *Hum. Exper. Toxicol.,* 18:95-105). RNA was cross-linked to the membranes using a UV Crosslinker (Stratagene, La Jolla, CA) and the membranes hybridized to random-primed cDNA probes encoding human CYP1A1. The cDNA probe for human CYP1A1 has previously been described (Shih *et al.* (1999) *Hum. Exper. Toxicol.,* 18:95-105). A cDNA probe for human 18S RNA probe (Ambion, Austin TX) was used to normalize the amount of RNA loaded in each lane. Hybridization of blots was performed as previously described (Quattrochi *et al.* (1985) *DNA,* 4:395-400). Autoradiographs of Northern blots were quantified by densitometry using a Model GS-670 Imaging Densitometer equipped with Molecular Analysis/Mac version 1.1.1. Image Analysis software (BioRad laboratories, Hercules, CA) or by scaning autoradiograms with a ScanMaker II (Microtek) and digitized with Un-Scan-It software (Silk Scientific, Orem Utah). Exposure times used were in the linear range of the film, Kodak XAR-5.

Data Analysis

Student's t test was used for the statistical analysis of data. Statistical significance was defined at a level of $p<0.05$. Data are expressed as the mean +/- standard deviation (SD).

1. Results

101L cells were plated at a density of 3.5×10^5 or 7.5×10^4 cells per well in twenty-four or ninety-six-well plates, respectively. Following exposure to the Ah receptor ligand, benzanthracene, luciferase activity was determined. When results obtained from ninety-six well plate assays were compared to those from twenty-four plates, negligible differences in luciferase activity were detected (**FIG. 17**). These findings alleviated the concern that too few cells per well would produce an inadequate signal. There was also concern that variability between replicate wells would be high. However, the ninety-six well format exhibited a maximum of 10% well-to-well variability with minimum background (**FIG. 17**). The following experiments were performed using the ninety-six well format.

The maximal time period for inducer exposure was determined by establishing a time course of inducer-mediated luciferase activity. Enhanced activity was observed within six hours of dosing with benzanthracene (100 micromolar), omeprazole (100 micromolar) or 3-MC (10 micromolar (**FIG. 18**). Maximum induction by benzanthracene (thirty-five fold) and 3-MC (fourteen fold) occurred at twelve hours while omeprazole mediated induction was maximal at eighteen hours (twelve fold), after which luciferase activity declined. The decline in inductive response was more than likely due to metabolism of the inducer by HepG2 CYP1A1. As expected, induction by rifampicin (100 micromolar) was negligible because this antibiotic is not known to be a CYP1A inducer (Kostrubsky *et al.* (1999) *Drug. Metab. Dispos.*, 27:887-894). These results indicate that this high-volume screening procedure is effective at monitoring easily detectable induction within a relatively short time period, for example less than twenty-four hours. In addition, the concentration dependent effects of various known CYP1A1 inducers were determined in this system. Dose response curves ranging from 0.5 to 2.5 nanomolar were generated for TCDD (**FIG. 19A**), and 1 to 200 micromolar for benzanthracene and omeprazole (**FIG. 19B**). For benzanthracene and omeprazole, maximum induction (thirty-five fold and twelve fold, respectively) occurred at 100 micromolar. The fold induction by TCDD had not peaked at a dose of 2 namomolar, and this dose produced a twenty-two fold increase in luciferase activity.

The use of high throughput methods for mechanistic studies were also investigated. To determine a mechanism that may be involved in flavonoid prevention of chemical carcinogenesis, the effects of several dietary flavonoids on CYP1A1 induction was examined. Results of these studies could indicate if the flavonoid exhibitied Ah receptor agonist and/or antagonist activities. Initial studies examined the ability of various naturally occurring flavonoids to induce CYP1A1-promoter-mediated reporter gene activity in the 101L cell line. Dose response curves for GTE, EGCG, quercetin, curcumin, kaempferol, naringenin, apigenin and resveratrol were determined. Of these flavonoids, resveratrol (10 micromolar) produced the largest induction of CYP1A1 (ten-fold). The second most effective flavonoid inducers were apigenin, uercetin and curcumin (three fold). A three-fold elevation in luciferase activity was observed with five micromolar of apigenin treatment, whereas higher doses of quercetin and curcumin (twenty micromolar) provided for similar levels of induction. Doses higher than five micromolar apigenin produced a decline in CYP1A1 induction, which more than likely is the result of cytotoxicity. GTE (0.1 milligrams/ml) (**FIG. 20**, inset) and kaempferol also produced slight induction (2 to 2.5 fold induction) on CYP1A1-promoter-mediated induction of luciferase activity at concentrations ranging from 1 to 20 micromolar (**FIG. 20**).

To validate similar inductive responses of the endogenous CYP1A1 gene, HepG2 cells were also treated with the same flavonoids. Enhanced CYP1A1 mRNA expression was observed in cells treated with GTE (10% of TCDD induction (**TABLE 2**). Although increased expression of CYPA1 mRNA occurred with these flavonoids, the induction was much less than that of beta-naphthoflavone (50% of TCDD response). Collectively, GTE, resveratrol and apigenin appear to be weak agonists for the Ah receptor.

TABLE 2

The Effect of Flavonoids on CYP1A1 mRNA Levels in HepG2 Cells

Treatment	HepG2 CYP1A1 mRNA (%) (a)
TCDD	100
50 micromolar beta-napthoflavone	50
100 micromolar beta-napthoflavone	53
Resveratrol	12
GTE	10
Apigenin	1
Naringenin	0
TCDD + Resveratrol	86
TCDD + Apigenin	43
TCDD + Naringenin	30
(a) CYP1A1 mRNA levels were normalized to TCDD induction (100% increase in CYP1A1 mRNA). Each value represents the mean of two separate determinations that differed by <10%.	

The ability of flavonoids to exhibit Ah receptor antagonism activity was also examined using this high throughput screening system. Co-treatment of the 101L cells with TCDD and flavonoids in the ninety-six well plate assay resulted in decreased TCDD-mediated induction of reporter gene activity by some of the flavonoids, indicating that certain of these dietary agents exhibited antagonist activity (**FIG. 21**). When the 101L cells were co-treated with GTE and TCDD a 58% reduction in luciferase activity was observed compared to cells treated with TCDD alone. Furthermore, the flavonoids naringenin and apigenin produced a 77% and 74% reduction, respectively, in TCDD mediated induction. Results of these studies demonstrate that these dietary flavonoids are capable of antagonizing TCDD-mediated induction of CYP1A1 promoter activity, with naringenin having the greatest effect (**FIG. 21**). Based on results where apigenin or

GTE alone displayed a 2.5 to 3-fold induction of CYP1A1-mediated reporter gene activity (**FIG. 20**), these flavonoids appear to exhibit agonist and antagonist activity toward the Ah receptor. The other flavonoids either produced no appreciable change in TCDD-mediated induction of luciferase activity or stimulated its effects. Indeed, co-treatment with TCDD and curcumin produced a 1.5-fold stimulation above the effects of TCDD alone, indicating that mechanisms in addition to those involving the AhR may play a role in induction of the P450 by curcumin. When HepG2 cells were co-treated with TCDD and individual flavonoids, results similar to those obtained with the reporter gene assay were observed. Resveratrol produced a slight decrease in the TCDD inductive response of CYP1A1 mRNA (14% reduction) whereas apigenin and naringenin produced significant reductions in CYP1A1 mRNA accumulation mediated by TCDD (57% to 70% decreases (**TABLE 2**). These results corroborate those produced in the 101L cell line and suggest that apigenin and naringenin have AhR antagonist activity.

To demonstrate if similar effects would occur in primary cultures of human hepatocytes, Northern analyses were performed on mRNA isolated from these cells trreated with TCDD, flavonoids, or a combination of TCDD and individual flavonoids. Results revealed similar findings to those produced by the high throughput or high volume screening system. Resveratrol enhanced CYP1A1 mRNA levels to 5% and 12% of TCDD induction in hepatocytes from two individual liver samples (Subject A and Subject C) while GTE enhanced CYP1A1 mRNA levels to 34% of TCDD induction in one culture (Subject A) (**TABLE 3**). In comparison, 100 micromolar benzanthracene caused induction of CYP1A1 mRNA to 50% of that observed with TCDD in all subjects. In hepatocytes from one subject, not only benzanthracene, but also resveratrol, apigenin and kaempferol produced accumulation of CYP1A1 mRNA (Subejct C, **TABLE 3**). Resveratrol increased expression to 12%, apigenin to 3% and kaempferol to 10% of that observed with benzanthracene. Hepatocytes from two other subjects (Subject B and Subject D, **TABLE 3**) did not display CYP1A1 induction with any of the flavonoids, but did exhibit CYP1A1 mRNA eccumulation produced by TCDD and benzanthracene (50% of TCDD levels). Human hepatocytes were also co-treated with TCDD and individual flavonoids. Resveratrol produced a 49% reduction in enhanced levels of CYP1A1 mRNA produced by TCDD. Apigenin and naringenin produced 78% and 80% reductions, respectively, in TCDD-mediated increases of

CYP1A1 mRNA (**TABLE 3**). These results were similar to those obtained from co-treatment of the 101L cell line with TCDD and apigenin or naringenin (**FIG. 21**)

TABLE 3

Effect of Flavonoids on CYP1A1 mRNA Levels in Primary Cultures of Human Hepatocytes

Treatment	Subject A (%) (a)	Subject B (%) (a)	Subject C (%) (b)	Subject D (%) (a)
TCDD	100	100	ND	100
Benzanthracene	50	50	100	52
GTE	34	0	ND	0
Resveratrol	5	0	12	0
Apigenin	ND (c)	0	3	0
Kaempferol	ND	0	10	0
Naringenin	ND	ND	ND	0
TCDD + Resveratrol	ND	ND	ND	51
TCDD + Apigenin	ND	ND	ND	22
TCDD + Naringenin	ND	ND	ND	20
(a)	CYP1A1 mRNA levels were normalized to TCDD induction (100% increase in CYP 1A1 mRNA)			
(b)	CYP 1A1 mRNA levels were normalized to benzanthracene induction (100% increase in CYP 1A1 mRNA			
(c)	ND indicates not determined			

Discussion

This example utilizes a reporter gene assay and a stable cell line, namely 101L cells (Postlind *et al.* (1993) *Toxicol. Apl. Pharmacol.,* 118:255-262), to screen potential CYP1A inducers. Stable cell lines harboring P450 enhancers and reporter genes are advantageous for screening applications because the need to continually transfect is alleviated, eliminating variability associated with transient transfections. Stably integratred cells also markedly increase sensitivity allowing induction to be easily assessed. Consistent results are obtained and the stable cells allow an alternative to other systems that are time consuming and labor intensive. Thus, the use of stable cell lines with P450 enhancers can facilitate screening of potential inducers. Indeed, the 101L reporter gene system is an application currently being used in 6 well plate formats by industry to screen environmental samples for the presence of CYP1A1-inducing compounds (Jones *et al.* (2000) *Environ. Toxicol. Pharmacol.,* 8:119-126).

To develop a high throughput system with stable cell lines, the previously characterized 101L cells were initially plated in either 24 well or 96 well plates having a standard footprint and treated with benzanthracene (**FIG. 17**). Results generated from these experiments indicated that the 96 well plate format was as efficient as the 24 well plate format. Furthermore, in the high throughput (96-well) format, there was minimum background and less than 10% well-to-well variability. In the presence of various CYP1A inducers, maximum induction (12 to 35-fold) occurred within a 24 hour exposure period, similar to that obtained in 6-well plates (Postlind *et al.* (1993) *Toxicol. Appl. Pharmacol.,* 118:255-262; Quattrochi and Tukey (1993) *Mol. Pharmacol.,* 43:504-508). Ziccardi *et al.* (*Toxicol. Sci.,* 54:183-193 (2000)) reported a 96 well format to screen serum samples for Ah receptor ligands.

To test the high throughput format of the present invention, additional CYP1A inducers were examined. A dose response curve was established for benzanthracene. Maximum induction in 101L cells previosuly reported in 6 well plates occurred at a dose of 50 micromolar benzanthracene (Jones *et al.* (2000) *Environ. Toxicol. Pharmacol.,* 8:119-126). The same dose produced maximum CYP1A1 mediated luciferase activity (33-fold) in the study described herein with the 96-well plate format (**FIG. 19B**). Other known CYP1A inducers including

3-methylcholanthrene, TCDD and omeprazole also produced induction of luciferase in the 96-well format whereas rifampicin, a CYP3A4 inducer, had no effect (**FIG. 17**), confirming the specificity of this system to respond solely to CYP1A inducers. TCDD and/or benzanthracene also induced CYP1A1 mRNA in HepG2 cells (**TABLE 2**) and in all human hepatocyte samples tested. Although not tested here, omeprazole has been shown in previous investigations to induce CYP1A's in human hepatocytes (Dias *et al.* (1990) *Gastroenterology,* 99:737-747 and Shih *et al.* (1999) *Hum. Exper. Toxicol.,* 18:95-105). Collectively, when an inducer produces greater than 12 fold increases in luciferase activity in the high throughput system (HTS), in all likelihood induction of CYP1A1 by the same agent would occur in human hepatocytes.

To determine if this HTS could be used to identify novel CYP1A1 inducing agents, the ability of a variety of dietary flavonoids to induce CYP1A1 was examined. Of the flavonoids examined, only resveratrol produced a substantial increase (10-fold) in CYP1A1 mediated-luciferase activity. However, cells treated with concentrations less than 20 micromolar resveratrol had negligible effects on luciferase activity, consistent with previous reports that this agent does not induce CYP1A1 mRNA in breast cancer cell lines or HepG2 cells (Ciolino *et al.* (1998) *Cancer Res.* 58:5707-5712, and Casper (1999) *Mol. Pharmacol.,* 56:784-790). When induction observed with the reporter gene assay was compared to CYP1A1 mRNA accumulation in primary hepatocytes and HepG2 cells, again resveratrol produced increases in CYP1A1 mRNA from HepG2 cells and in hepatocytes from two individuals (**TABLE 2**, **TABLE 3**, particularly Subject A and Subject C). These results indicate that agents producing 10-fold increases in luciferase activity observed in the HTS, could also produce CYP1A1 induction in hepatocytes. Those flavonoids producing 2.5-fold induction or greater in the HTS system, namely GTE and apigenin, also produced slight increases in the accumulation of CYP1A1 mRNA in primary hepatocytes isolated from one of three individuals examined here. Similarly, kaempferol which produces two-fold increases in luciferase activity also caused accumulation of CYP1A1 mRNA in hepatocytes from a single individual. In contrast, quercetin and curcumin did not elicit induction of CYP1A1 mRNA in isolated hepatocytes (data not shown), but did produce moderate increases (2.5 to 3-fold) in luciferase activity. Thus, this disparity in results between the HTS and human hepatocytes among various agents, suggests that when reporter

assays exhibit relatively low levels of induction by a particular agent (for example, 2 to 3 fold), increases in primary hepatocyte CYP1A1 may or may not occur.

Based on results obtained here with the HTS, less than 2-fold induction of luciferase activity indicates that increased expression of CYP1A1 would be unlikely to occur in primary hepatocytes. The importance of the hepatocyte finding corroborating those of the HTS lies in the ability to extrapolate human hepatocyte data to the in vivo situation (Ito *et al.* (1998) *Annu. Rev. Pharmacol. Toxicol.*, 38:461-499, and Kedderis (1997) *Chem. Biol. Interact.*, 107:109-121). For example, omeprazole produced induction of CYP1A's in both isolated human hepatocytes (Shih *et al.* (1999) *Hum. Exper. Toxicol.*, 18:95-105, and Diaz *et al.* (1990) *Gastroenterology*, 99:737-747) and *in vivo* (Rost *et al.* (1992) *Clin. Pharmacol. Ther.*, 52:170-180). In general, the pharmacokinetics of xenobiotics have been well predicted from studies with isolated hepatocytes (Kedderis (1997) *Chem. Biol. Interact.*, 107:109-121). In this example, good agreement occurred between results generated in the stably transfected cells and human liver cells (primary hepatocytes and HepG2 cells), suggesting that cell lines stably transfected with CYP enchancers would be able to predict the *in vivo* outcome.

The HTS format for assessing CYP1A1 induction is useful in identifying agents that can elevate expression of CYP1A1 by way of the Ah receptor. Furthermore, this system can be used to determine mechansims involved in CYP induction. This example demonstrates that certain flavonoids were identified as exhibiting weak agonist and/or antagonist activity towards the Ah receptor. With regards to the reliability of this HTS for identifying CYP inducers, signal to noise ratios were low and well-to-well and replicate variability were below 10% allowing induction to be readily detected in this system. Also, results generated with this HTS reflected inducer responses obtained in isolated human hepatocytes or HepG2 cells.

EXAMPLE III

Nonhuman Nucleic Acid Molecules Encoding Proteins Involved In Drug Metabolism

Nonhuman organisms, such as but not limited to primates, can be a source of nucleic acid molecules encoding proteins involved in drug metabolism and the proteins encoded thereby. These nucleic acid molecules and proteins can be used in and are part of the present invention. The nucleic acid molecules can encoded any protein involved in drug metabolism, including, but not limited to CYP proteins, PXR, and CAR. These nonhuman nucleic acid molecules and proteins, and allelic variants thereof, can be used in place of their human counterparts in the methods of the present invention. For Example, in **Example I** above, nonhuman PXR can be used in place of human PXR, and in **Example II** above, nonhuman CYP1A1 can be used in place of human CYP1A1. Nonhuman nucleic acids and peptides can be identified using methods known in the art, such as sequence homology screening using appropriate algorithms. Such algorithms are available, including BLAST (www.ncbi.nlm.nih.gov/BLAST)and ClustalW (www.ebi.ac.uk/clustalw). Alternatively, PCR primers can be identified for the human nucleic acid molecule or consensus sequences identified and used to amplify clone and isolate the nonhuman nucleic acid molecule and protein counterpart using known methods. In addition, nonhuman sequences can be used to identify human nucleic acid molecules encoding proteins involved in drug metabolism and the encoded proteins. These nucleic acid molecules and proteins are useful in the methods of, and are part of, the present invention.

The encoded proteins of the nucleic acid molecules can be expressed, isolated, and characterized using methods known in the art, such as methods described in Sambrook *et al.*, Molecular Biology: A Laboratory Manual (Third Edition), Cold Spring Harbor Press, New York (2001).

The activity of the proteins can be validated and confirmed using methods known in the art and described herein. In particular the cell based assays described herein, can be used to validate and confirm the activities of the proteins.

I. Cloning of Monkey PXR

PXR plays an active role in drug metabolism by inducing regulation of CYP3A4 expression. Alternatives to human PXR (hPXR) can be used in the present invention, such as non-human PXR, including nucleic acid molecules and peptide or proteins. Allelic variants of such human and non-human PXR can also be used in and are part of the present invention. One alternative to hPXR is Monkey PXR (mkPXR), which was isolated as described herein.

cDNA synthesis: Total RNA 2 microgram from monkey liver was used as a template for first strand of cDNA synthesis that mRNA was reverse transcribed to cDNA by incubating SuperScript II RT in a buffer containing total RNA, 1x PCR buffer, 0.5 microgram oligo(dT), 2.5 milimolar $MgCl_2$, 0.5 micromolar dNTP, 10 milimolar DTT (Life Technologies, Gaithersburg, MD), generally following the manufacturer's instructions.

PCR reaction: PCR reactions were performed with 1 microliter of the first strand reaction in 25 microliter final volumes containing 2.5 mircroliter 10x PCR buffer (Stratagene), 0.2 milimolar of each dNTP, 0.4 micromolar of each primer which is consensus sequence between human, rat and mouse PXR (sense 5' gattgttcaaagtggacccca 3' (**SEQ ID NO. 5**); reverse 5' tgtccttcctgaggaatgcta 3' (**SEQ ID NO. 6**)), and 1 unit of Taq polymerase (Stratagene). The PCR reaction was performed in a Perkin Elmer 2400 thermocycler and was incubated at 94°C for 3 min, followed by 35 cycles of 94°C for 20 s, 58°C for 30 s and 72°C for 90 s. Cycling was followed by a final extension of 7 min at 72°C. The PCR product, 1.5 kb approximately, was ligated into PCR2.1 vector and sequenced by appropriate methods known in the art. The homology between human and mkPXR is 95.96% for open reading frame. A pair of Oligos (5' atggaggtgagacccaaagaa 3 (**SEQ ID NO. 7**)'; 5' tcagctaccccgtgatgccgaa 3' (**SEQ ID NO. 8**)) was used to amplify open reading frame of mkPXR in PCR, which was ligated into expression vector pIRESneo.

The mkPXR nucleic acid sequence is:

5'ctggaggtgagacccaaagaaggctggaaccatgctgactttgtatactgtgaggacacaga
gtttgctcctggaaagcccactgtcaacgcagatgaggaagttgggggtccccaaatctgccgt
gtatgtggggacaaggccactggttatcacttcaatgtcatgacatgtgaagggtgcaagggctt
tttcaggagggccatgaaacgcaacgcccgccttaggtgccccttccggaagggcgcctgcg
agatcacccggaagacccggcgacagtgccaggcctgccggctgcgcaagtgcctggagag
cggcatgaagaaggagatgatcatgtccgacgcggccgtagaggagaggcgggccttgatc
aagaggaagaaaagagaacggatcgggactcagccacccggagtgcaggggctgacggag
gagcagcggatgatgatcagggagctgatggacgctcagatgaaaacctttgacactaccttct
cccatttcaagaatttccggctgccaggggtgcttagcagtggctgtgagatgccagagtctctg
caggccccatcgagggaagaagctgccaagtggaaccaggtcaggaaagatctgtggtctgt
gaaggtctccgtgcagctgcgggggggaggatggcagtgtctggaactacaaacccccagccg
acaatggcgggaaagagatcttctccctgctgccccacatggctgacatgtcaacctacatgttc
aaaggcatcatcaactttgccaaagtcatctcctacttcagggacctgcccattgaggaccagat
ctccctactgaaggggccacttttgagctgtgccagctgagattcaacacagtattcaacgtgg
agactggaacttgggagtgtggccggctgtcctactgcttggaagaccctgcaggtggtttcca
gcaacttctgctggagcccatgctgaaattccactacatgctgaagaagctgcagctacacgag
gaggagtatgtgctgatgcaggccatctccctcttctccccagaccgcccaggtgtggtgcagc
accacgtggtggaccagctgcaggagcaatacgctattactctgaagtcctacattgaatgcaat
cggccccagcctgctcataggttcctgttcctgaagatcatggctatgctcaccgagctccgcag
catcaacgcccagcacacccagcggctgctgcgcatccaggacatacacccctttgctacgcc
cctcatgcaggagttgttcggcatcacgggtagctga3' (**SEQ ID NO. 9**)).

The deduced amino acid sequence for mkPXR is:

LEVRPKEGWNHADFVYCEDTEFAPGKPTVNADEEVGGPQI
CRVCGDKATGYHFNVMTCEGCKGFFRRAMKRNARLRCPF
RKGACEITRKTRRQCQACRLRKCLESGMKKEMIMSDAAVE
ERRALIKRKKRERIGTQPPGVQGLTEEQRMMIRELMDAQM
KTFDTTFSHFKNFRLPGVLSSGCEMPESLQAPSREEAAKWN
QVRKDLWSVKVSVQLRGEDGSVWNYKPPADNGGKEIFSLL
PHMADMSTYMFKGIINFAKVISYFRDLPIEDQISLLKGATFEL
CQLRFNTVFNVETGTWECGRLSYCLEDPAGGFQQLLLEPML
KFHYMLKKLQLHEEEYVLMQAISLFSPDRPGVVQHHVVDQ
LQEQYAITLKSYIECNRPQPAHRFLFLKIMAMLTELRSINAQ
HTQRLLRIQDIHPFATPLMQELFGITGS (**SEQ ID NO. 10**)).

The mkPXR can be used in experiments instead of hPXR similar to Example I above where mkPXR expressing cells contain a luciferase reporter. HepG2 cells are transfected with the mkPXR and treated, and then colonies of stable cells are tested for inducible luciferase activity by treatment with compounds of interest.

II. Cloning of Monkey CAR

CAR plays an active role in drug metabolism. Alternatives to human CAR (hCAR) can be used in the present invention, such as non-human CAR, including nucleic acid molecules and peptide or proteins. Allelic variants of such human and non-human CAR can also be used in and are part of the present invention. One alternative to hCAR is Monkey CAR (mkCAR), which was isolated as described herein.

cDNA synthesis: Total RNA 2 microgram from monkey liver was used as a template for first strand of cDNA synthesis that mRNA was reverse transcribed to cDNA by incubating total RNA in a buffer containing SuperScript II RT, 1 x PCR buffer, 0.5 microgram oligo (dT), 0.5 milimolar dNTP, and 10 milimolar DTT (Life Technologies, Gaithersburg, MD), generally following the manufacturer's instruction.

PCR reaction: PCR reactions were performed in a 25 microliter final volume of 1 microliter of first strand reaction, 2.5 microliter 10 x PCR buffer, 0.2 milimolar of each dNTP, 0.4 micromolar of a pair of primers which are highly consensus sequences between human and mouse (sense 5' atgacagccaccccaacacgt 3'(**SEQ ID NO. 11**); reverse 5' aaggaagtgagcatggcctca 3' (**SEQ ID NO. 12**)), and 1 unit of Taq polymerase (Stratagene). The PCR reaction was performed in a Perkin Elmer 2400 thermocycler and was incubated at 94°C for 3 min, followed by 35 cycles of 94°C for 20s, 58°C for 30s, and 72°C for 90s. Cycling was followed by a final extension of 7 min at 72°C. The PCR product, 1.1 killobases approximately, was ligated into PCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced by appropriate methods known in the art. The homology between human and monkey CAR is 96.49 % for open reading frame. Monkey CAR then was cut out from PCRII vector by EcoRI and ligated into EcoRI-digested expression vector pIRESneo (Clontech, Palo Alto, CA).

The mkCAR nucleic acid sequence is:

5'atgacagccaccccaacacgtgatgtcatggccagtagggaagatgagctgaggaactgtgtggtatgt
ggggaccaggccacaggctaccacttcaacgcgctgacttgtgagggctgcaagggtttcttcaggagaa
cagtcagcaaaagcattggtcccacctgccccttgctggaagctgtgaagtcagcaagattcagaggcgc
cactgcccagcctgcaggttgcagaagtgcttagatgctggcatgaggaaagacatgatactgtcggcag
aagccctggcattgcggcgagcaaagcaggcccagcggcgggcacagcaaacacctatgcaactgagt
aatgagcaagaagagttgatccagacactcctgggggcccacacccgccacatgggcaccatgtttgaac
agtttgtgcagtttaggcctccagctcatctgttcatccatcaccagcccttgcccaccctggcgcctgtgctg
cctctggtcacacacttcgcagacgtcaacacgttcatggtacagcaagtcatcaagtttaccaaggacctg
cctgtcttccgttctctgcccattgaagaccagatctcccttctcaagggagcagctgtggaaatctgtcatat
cgtactcaataccactttctgtctccaaacacaaaacttcctctgcgggcctcttcgctacacaattgaagac
gcagcccgtgtatctcccgcagtggggttccaggtagagtttttggagttgctctttcacttccatggaacact
acgaaaactgcagctccaggagcctgagtatgtgctcttggctgccatggccctcttctctcctgaccgacc
tggagttacccagagacatgagattgatcagctgcaagaggagatggcactgactctgcaaagctacatca
agggccagcagcaaaggccccgggatcggtttctgtatgcgaagttgctgggcctgctggctgagctccg
gagcattaatgaggcctacgggtaccaaatccagcacatccagggcctgtctgccatgatgccattgctcc
aggagatctgcagctgaggccatgctcacttcctt3' (**SEQ ID NO. 13**).

Deduced amino acid sequence for mkCAR is:

MTATPTRDVMASREDELRNCVVCGDQATGYHFNALTCEGCKGFF
RRTVSKSIGPTCPFAGSCEVSKIQRRHCPACRLQKCLDAGMRKDMI
LSAEALALRRAKQAQRRAQQTPMQLSNEQEELIQTLLGAHTRHMG
TMFEQFVQFRPPAHLFIHHQPLPTLAPVLPLVTHFADVNTFMVQQV
IKFTKDLPVFRSLPIEDQISLLKGAAVEICHIVLNTTFCLQTQNFLCGP
LRYTIEDAARVSPAVGFQVEFLELLFHFHGTLRKLQLQEPEYVLLA
AMALFSPDRPGVTQRHEIDQLQEEMALTLQSYIKGQQQRPRDRFL
YAKLLGLLAELRSINEAYGYQIQHIQGLSAMMPLLQEICS (**SEQ ID NO. 14**)).

The mkCAR can be used in such experiments similar to Example I above where cell lines such as Cell line HepG2 are stably transfected with mkCAR and grown in media and treated. The cells are then exposed to compounds of interest followed by determining luciferase activity. The result obtained is then compared with HepG2 cells to validate similar inductive responses with the test compound of interest.

EXAMPLE IV

Identification of a Constitutive Androstane Receptor (CAR) Regulatory Element in the CYP2C19 Gene

The CYP2C subfamily of P450 enzymes is an important class of enzymes composed of CYP2C9, CYP2C19, and CYP2C8 that are believed to metabolize approximately 20% of known therapeutic agents (Ingelman-Sundberg *et al.* (1999) *Trends Pharmacol. Sci.*, 20:342-349). Of these isoforms, CYP2C19 has been implicated in producing inter-individual variability to certain drug substrates. One factor involved in the wide variation of metabolism among individuals by this P450 is its inducibility. CYP2C19 is induced by phenobarbital (PB) and rifampicin (RIF), is induced to a greater extent by phenobarbital than by rifampicin when compared to CYP2C8 and CYP3A4, and thus may be regulated by different mechanisms from the latter (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482).

The expression of CYP2C8 and CYP3A4 was enhanced by RIF in primary cultures of human hepatocytes (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482). Rifampicin's induction of CYP3A4 is mediated by PXR (Goodwin *et al.* (2002) *Annu. Rev. Pharmacol. Toxicol.*, 42:1-14; Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023). Expression of CYP2C enzymes may be mediated by PXR or other receptors such as the orphan nuclear receptor, CAR (constitutive androstane receptor). CAR has been shown to bind to a specific DNA response element of CYP2B (Honkakoski *et al.* (1998) *Mol. Cell. Biol.*, 18:5652-5658), which is also recognized by PXR (Xie *et al.* (2000) *Genes Dev.*, 14:3014-3023). CYP2C9 expression was shown to be governed by CAR; a region of the CYP2C9 gene between -2900 to -2841 base pairs upstream of the translational start site was found to be CAR-responsive (Ferguson *et al.* (2002) *Mol. Pharmacol.*, 62:737-746). This region consists of two DR-5 nuclear receptor-binding motifs capable of binding hCAR, mCAR and to a lesser extent, hPXR. The majority of binding and hCAR activation was derived from the NR1 portion of the CAR-response element. While phenobarbital was capable of increasing the CYP2C9 response in HepG2 cells transfected with human CAR (hCAR), hCAR did not confer drug inducibility to the

CAR-response element in a reporter gene assay. Thus, constitutive regulation of CYP2C9 by hCAR was demonstrated but the hCAR response element did not account for phenobarbital inducibility of the gene.

In contrast to classical nuclear receptors, human CAR is transcriptionally active in the absence of ligand (Shiraki *et al.* (2003) *J. Biol. Chem.*, 278:11344-11350; Ueda *et al.* (2002) *Mol. Pharmacol.*, 61:1-6). Phenobarbital activates hCAR through an indirect mechanism wherein following phenobarbital exposure, hCAR translocates from the cytoplasm to the nucleus and forms a heterodimer with RXR. This heterodimer binds a 51 base pair distal element, Phenobarbital-Responsive Enhancer Module (PBREM) that contains two NR binding sites that are direct repeat (DR)-4 motifs (Sueyoshi and Negishi (2001) *Annu. Rev. Pharmacol. Toxicol.*, 41:123-143; Honkakoski *et al.* (1998) *Mol. Cell. Biol.*, 18:5652-5658). This PBREM sequence was found in CYP2B6 and other phenobarbital responsive genes. Because CAR exhibits an intrinsically high transcriptional activity, nuclear localization of the receptor results in target gene expression in the absence of ligand binding. Significant overlap in the pharmacology of hCAR and PXR and lack of an *in vitro* system to study CAR function have hindered hCAR characterization. A recent report describes a novel hCAR agonist, 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorbenzyl)oxime (CITCO) (Maglich *et al.* (2003) *J. Biol. Chem.*, 278:17277-17283), which induces human CAR translocation and the CAR target gene CYP2B6 in human hepatocytes, but produces only a weak response in immortalized tumor cells.

Phenobarbital is a better inducer of CYP2C19 in human hepatocytes than was rifampicin or dexmethasone (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482). Studies were designed to determine if CAR was involved in the mechanism of drug-mediated induction of CYP2C19. The results identified a nuclear receptor binding site in the CYP2C19 gene which confers xenobiotic responsiveness to CYP2C19 in a reporter gene assay using stable cell lines containing hCAR. CAR was shown to induce HepG2 CYP2C19 by phenobarbital and by CITCO in cell lines that have been stably transfected with hCAR.

Materials. The cDNA probe to human 18s rRNA was purchased from Ambion (Austin, TX). Restriction enzymes were purchased from New England Biolabs (Beverly, MA), and bicinchoninic acid was from Pierce Chemical Co. (Rockford, IL). Rifampicin, methoxychlor, dexamethasone, and phenobarbital were purchased from Sigma Chemical Co. (St. Louis, MO). 6-(4-chlorophenyl) imidazo [2,1-*b*][1,3] thiazole-5-carbaldehyde O- (3,4-dichlorbenzyl)oxime (CITCO) was obtained from Biomol (Plymouth Meeting, PA). Culture dishes were from VWR (Westchester, PA) and fetal bovine serum (FBS) was from Hyclone (Logan, UT). Dulbecco's Modified Eagle Medium (DMEM) and lipofectamine 2000 were purchased from Life Technologies (Gaitherburg, MD). RNeasy RNA purification and plasmid purification kits were obtained from Qiagen (Valencia, CA), and nylon membranes were purchased from Molecular Simulations, Inc. (Westboro, MA). HepG2 cells stably integrated with human CAR (Hep-CAR) were a generous gift from Puracyp, Inc. (Carlsbad, CA). All other reagents used were of the highest quality available.

Plasmid Construction and Cloning of CYP2C19 Promoter Region. The full-length coding regions of human PXR, GR, RXRa, and CAR were obtained by RT-PCR using methods that were previously described (Carpenter *et al.* (1996) *Mol. Pharmacol.,* 49:260-268; Raucy (2003) *Drug Metab. Dispos.,* 31:533-539). Briefly, the 1300 base pair amplified hPXR, 2331 base pair hGR, 1377 base pair hCAR, and 1391 base pair RXRa products were cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and subjected to sequence analysis. The sequences obtained agreed over the entire coding region with those previously described (Baes *et al.* (1994) *Mol. Cell. Biol.,* 14:1544-1552; Hollenberg *et al.* (1985) *Nature,* 318:635-641; Lehmann *et al.* (1998) *J. Clin. Invest.,* 102:1016-1023; Mangelsdorf *et al.* (1990) *Nature,* 345:224-229). The cDNAs were then excised from pCR2.1 by digestion with BamHI and NotI and cloned into analogous sites of a pIRESneo vector (Clontech, Palo Alto, CA). The CYP2C19 enhancer region containing 15 kilobases upstream of the translational start site (Ensembl Human Genome Website, www.ensembl.org/Homo_sapiens/) was initially separated into 2000 to 3000 base pair segments and the most proximal and most distal segments from the translation start site were integrated into a luciferase vector (pGL3 promoter, Promega, WI). The proximal 2000 base pair fragment was identical to that previously reported (Arefayene *et al.* (2003) *Pharmacogenetics,*

13:199-206). Once the segment exhibiting the highest activity in the presence of hCAR was identified, it was further subdivided into 12 sections as shown in **TABLE 4**, cloned into pGL3 promoter (Promega, WI), and transiently transfected into Caco2 cells to assess transcriptional activation.

Cell Culture, Transfection, and Treatment of Cell Lines. HepG2 cells or HepG2-derived transformed cell lines harboring hCAR cells were grown as monolayers in DMEM (Gibco/BRL, Gaithersburg, MD) containing 50 units per milliliter penicillin, 100 milligrams per milliliter streptomycin, 0.1 millimoles per liter non-essential amino acids (Gibco/BRL), and 10% fetal bovine serum (FBS, Hyclone, Logan, UT), and maintained in an atmosphere of 5% CO_2 and 95% air at 37 degrees Celsius. Cells were seeded in T75 flasks and grown to confluency. Passage of the stable cells was limited to 30. Upon reaching 75% confluency, cells were removed from flasks by trypsinization and replated in 24-well plates at a density of 3×10^6 cells per well in DMEM medium containing 10% FBS. Plasmid DNA used in transfection experiments was prepared by purification on Qiagen columns. Reporter constructs, pRL-sv40 (to control for transfection efficiency), hCAR, and control vectors were introduced into HepG2 cells obtained from the American Type Culture Collection (Manassas, VA). Hep-CAR cells received pRL-sv40, CYP2C19-IR4 reporter construct, or control vectors; hGR was also added to some cells. Hepatoma cells were transfected with lipofectamine 2000 reagent according to manufacturer's procedures. For transfection, medium was replaced with DMEM lacking FBS. HepG2 cells received 500 nanograms CYP2C19-reporter construct or 500 nanograms of control vector, 250 nanograms hCAR, vector, and 10 nanograms pRL-sv40. Hep-CAR cells received 500 nanograms CYP2C19-IR4, 10 nanograms pRL-sv40, 100 nanograms hGR, and control vectors. Five hours following transfection, 1 milliliter of DMEM containing 20% FBS was added to each well to create a final FBS concentration of 10%. The culture medium was removed after 24 hours of incubation with the lipofectamine-DNA complexes and fresh media containing 10% dextran-treated FBS containing drug or natural product was added. Control cells received media with 0.1% dimethylsulfoxide (DMSO) and cells were treated for 48 hours with media changes at 24 hours.

Transcriptional Activation Assays. Following transfection and treatment, the hepatocytes or HepG2 cells were rinsed in phosphate-buffered saline (PBS) and harvested by adding 100 milliliters per well of 1X passive lysis buffer contained in a dual-luciferase reporter assay kit (Promega, Madison, WI). Luciferase activity of cell lysates was then determined using a Lumistar galaxy luminometer (BMG Labtechnologies, Offenburg, Germany). Firefly luciferase activities were determined from two independent transfections and normalized against *Renilla luciferase* activities of the internal control pRL-sv40 vector obtained from the same culture. Results were expressed as relative light units or fold increase above control (DMSO-treated cells) ± standard deviation.

Gel Mobility Shift Assays. Electrophoretic gel mobility shift assays were performed. Briefly, human RXR, human CAR, and human PXR were synthesized *in vitro* using the TNT Quick-Coupled In Vitro Transcription/Translation system (Promega), following the manufacturer's protocol. Probes were labeled with [gamma-^{32}P]dCTP and the probe was purified by Microspin G-25 columns (Amersham Biosciences). A volume of 150,000 counts per minute of labeled probe was applied to each binding reaction in 10 moles per liter HEPES, pH 7.6, 0.5 moles per liter dithiothreitol, 15% glycerol, 0.05% Nonidet P-40, 50 millimoles per liter NaCl2, 2 milligrams of poly(dI-dC), and 1 to 2 milliliters of *in vitro* transcribed/translated proteins to a final reaction volume of 10 milliliters. The reactions were incubated at room temperature for 20 minutes, then loaded on 5% acrylamide gels in Tris/acetate/EDTA buffer, dried, and exposed to film for 6 to 18 hours at –70 degrees Celsius.

RNA Isolation and Northern Blot Analysis. Hepatocyte RNA was prepared using RNeasy kits, and quantified by measuring the absorbance at 260 nanometers; purity was assessed from the 260/280 nanometer absorbance ratio and by integrity of the 28s and 18s bands on agarose gels. Total RNA (10 micrograms) was subjected to electrophoresis on 1% agarose-2.2 moles per liter formaldehyde gels, followed by transfer to nylon membranes (Shih *et al.* (2000) *Arch. Biochem. Biophys.*, 373:287-294). RNA was bound to the membranes using a Stratalinker ultraviolet crosslinker (Stratagene, La Jolla, CA), after which the membranes were hybridized with random-primed ^{32}P-labelled cDNA probes encoding human CYP2C19 or 18s rRNA. The CYP2C19 probe was previously described (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482,

which is herein incorporated by reference). Hybridization conditions have been described elsewhere (Allen *et al.* (2001) *Drug Metab. Disp.*, 29:1074-1079; Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482, which are herein incorporated by reference). DNA-RNA hybridization signals were measured on autoradiograms with a ScanMaker II flat-bed scanner (Microtek, Redondo Beach, CA), and the signal intensities integrated using Un-Scan-It software (Silk Scientific, Orem, UT). Hybridization signals obtained with a human 18s rRNA probe (Ambion, Austin, TX) were used to normalize the amounts of RNA loaded onto the gels.

Western Blot Analysis. Protein concentrations were determined with the bicinchoninic acid (BCA) procedure using bovine serum albumin as the standard (Smith *et al.* (1985) *Anal. Biochem.*, 150:76-85). Western blotting of hepatocyte microsomal proteins to nitrocellulose, and subsequent immunochemical staining with 200 milligrams of anti-CYP2C19 IgG was performed as described elsewhere (Lasker *et al.* (1998) *Arch. Biochem. Biophys.*, 353:16-28). The properties of the anti CYP2C19 polyclonal antibodies used for these studies have been reported earlier (Lasker *et al.* (1998) *Arch. Biochem. Biophys.*, 353:16-28; Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482; and Wester *et al.* (2000) *Drug Metab. Dispos.*, 28:354-359, which are herein incorporated by reference). HepG2 CYP2C19 enzyme levels were quantified by first scanning the blots with the ScanMaker II scanner, and then integrating immunostaining intensities with Un-Scan-It software. Results were presented as the mean ± standard deviation in the case of two or more samples.

RESULTS

Transcriptional Activation of 5' CYP2C19 Fragments. Fragments were generated that complement various upstream regions of the CYP2C19 gene (**TABLE 4**). These fragments correspond to regions that are –15904 to –12584 base pairs (fragment 2C19-76) and –2786 to –84 base pairs (fragment 2C19-77) upstream from the translational start site. Fragments were generated by PCR and cloned into the pGL3 promoter vector (containing luciferase as a reporter). Caco-2 cells were transiently transfected with each of the CYP2C19 fragments in the presence and absence of hCAR. Forty-eight hours following transfection, luciferase activity was

determined for each of the fragments to indicate levels of transcriptional activation of CYP2C19 fragments, as shown in **TABLE 4**.

TABLE 4

Positions and Transcriptional Activation of CYP2C19 Regulatory Region Fragments

Fragment	Length (in base pairs)	Position from translation start site of CYP2C19	Fold increase above no receptor (luciferase activity)
2C19-76	3320	-15904 to -12584	1.5
2C19-77	2700	-2786 to -84	2.67
3A1	970	-2604 to -1634	4.08
3A2	860	-1954 to -1094	2.31
3A4	880	-964 to -84	0.98
3A6	325	-1959 to -1634	2.24
F1	377	-2341 to -1964	1.09
F2	332	-1939 to -1607	6.5
F3	87	-1977 to -1890	1.29
F4 **(SEQ ID NO. 15)**	26	-1895 to -1870	115.5
F5	186	-1876 to -1690	?
F6	95	-1710 to 1615	1.39
CYP2B6 (PBREM)	---	---	6.16

Transient transfection into Caco-2 cells of hCAR and constructs generated with fragments 2C19-77 and 2C19-76 and the luciferase reporter gene revealed that fragment 2C19-77 exhibited the greatest activity, 2.67-fold greater than cells receiving only the reporter construct (no receptor). Subdivisions of fragment 2C19-77 revealed that fragments 3A1, 3A2, and 3A6 contained response elements permissive to hCAR activation and that exhibited luciferase activities 4.08-, 2.31-, and 2.24-fold greater, respectively, than that observed for cells in the absence of hCAR. Further deletions within 3A1 to produce F1 and F2 revealed that F2 exhibited luciferase activity that was similar to that of the PBREM of CYP2B6, 6.5- and 6.2-fold, respectively, in the presence of hCAR. Subdividing F2 into four additional fragments, F3, F4, F5, and F6, revealed that F4, with the sequence AACCAAACTCTTCTGACCCCCAATCT (**SEQ ID NO. 15**), exhibited the greatest relative increase in luciferase activity in the presence of hCAR when

compared to activity in the absence of the receptor (115.5-fold) (**Table 1**). Fragments F3 and F6 exhibited slight activity in the presence of hCAR when compared to activity in the absence of the receptor.

Element Identification and Gel Shift Analysis of Binding. To specifically examine the potential of the F4 fragment (**SEQ ID NO. 15**) to regulate CYP2C19 through hCAR, gel shift analyses were performed. The 26 base pair fragment (**FIG. 22**) was examined for its ability to bind the transcription factors hCAR and hPXR. This site resembles imperfect AGGTCA (**SEQ ID NO. 16**) motifs known for many nuclear receptor binding sites, including CAR and PXR. To test the binding capabilities of this putative regulatory module, oligonucleotide probes to the F4 and F5 modules were designed, the double stranded segment labelled, and binding assays performed with *in vitro* transcribed/translated nuclear receptors, hCAR and hPXR. These binding studies produced an intense band with hCAR/hRXR proteins that was not observed with hCAR alone and was competed out by addition of 5-fold excess cold competitor double-stranded oligonucleotide. Similar but somewhat lower intensity binding was observed for hPXR with the F4 fragment (**SEQ ID NO. 15**). **FIG. 23** depicts a representative example of the results of these analyses. Here, the CYP2B6 DR4 sequence was used as a positive control and demonstrates strong binding with hCAR/hRXR, whereas hPXR/hRXR binds with significantly lower affinity, consistent with published reports that CAR and PXR can bind to the identical enhancer elements (Xie *et al.* (2000) *Genes Dev.*, 14:3014-3023). This binding was lost by competition with 5-fold excess cold competitor. By comparison, the F4 fragment (**SEQ ID NO. 15**) from CYP2C19 also binds strongly to hCAR/hRXR and more weakly to hPXR/hRXR. For comparison, PXRE from CYP3A1 was used as a positive control for hPXR/hRXR. The binding of hPXR exhibits much greater intensity than does binding to hCAR/hRXR. These regions were between –1891 to –1876 for CAR. In addition, a glucocorticoid responsive element (GRE) between –1750 to –1736 was identified.

EXAMPLE V

High Throughput Screening to Assess the Effect of Herbal Supplements and Environmental Chemicals on Drug-Metabolizing Enzymes

The aryl hydrocarbon receptor (AhR) and the pregnane X receptor (PXR) regulate metabolic pathways in response to chemical exposure by altering gene expression patterns of drug-metabolizing enzymes (DMEs), which may influence drug-drug interactions and drug or carcinogen activation and detoxification. AhR belongs to the PAS family of transcription factors, and is stimulated by environmental contaminants such as polycyclic aromatic hydrocarbons (PAHs) and their halogenated derivatives, triggering the transcriptional activation of the CYP1 family (CYP1A1, CYP1A2, and CYP1B1) (Postlind *et al.* (1993) *Toxicol. Appl. Pharmacol.*, 118:255-262). Induction of CYP1 family enzymes, which catalyze the metabolic activation of xenobiotics, has been associated with carcinogenesis. CYP1As are induced by PAHs and by other compounds, including indole-3,2-b-carbazole, curcumin, tryptophan, and bilirubin.

Over-the-counter remedies, including herbal medicine and dietary supplements, are a potential source of xenobiotics. Use of herbal medicines, particularly when combined with conventional drugs, poses a risk to the public of drug-drug interactions. For example, St. John's Wort (*Hypericum perforatum*), one of the most commonly used herbals, has been shown to induce cytochrome P450 3A4 (CYP3A4), an enzyme which interacts with numerous conventional drugs and reduces their efficacy. Hyperforin, a constituent of St. John's Wort, is a potent ligand for the pregnane X receptor (PXR), a nuclear receptor that activates target genes such as CYP3A4 (Moore *et al.* (2000) *Proc. Natl. Acad. Sci. U.S.A.*, 97:7500-7502). PXR has been proposed to act as a transcriptional regulator in response to a number of steroids and xenobiotics and to mediate the induction of CYP3A4 gene expression (Blumberg *et al.* (1998) *Genes Dev.*, 12:3195-3205; Kliewer *et al.* (1998) *Cell*, 92:73-82; Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023). PXR forms a heterodimer with 9-*cis* retinoic acid receptor (RXR), and the heterodimer's binding site has been mapped to a conserved sequence within the promoter region that contains two half sites of AG(T/G)TCA (**SEQ ID NO. 17**) and is organized as an

inverted repeat separated by six base pairs (an IR-6 motif), or organized as a direct repeat with a 3 base pair spacer (a DR3 motif) (Xie *et al.* (2000) *Nature,* 406:435-439).

Gene expression profiling of drug metabolizing enzymes in response to xenobiotic exposure has almost exclusively depended upon the use of human tissues and primary hepatocyte cultures. These assays are limited by the availability and the inter-individual difference of the donors. Cell-based bioassays of the present invention are capable of identifying xenobiotics that induce receptor (for example AhR or PXR) mediated genes, including CYP1A1, CYP1A2, and CYP3A4, in a high-throughput system.

Stable cell lines were developed that incorporated a target response element, corresponding to a nuclear receptor, into a luciferase reporter plasmid. When the receptor is activated in response to ligands, it is subsequently translocated to the nucleus where it acts as a transcription factor, binding to the corresponding response element and turning on transcription of the reporter gene (luciferase). Botanicals and environmental compounds possessing the ability to activate the expression of target drug metabolizing enzymes through either the Ah or PXR receptor were identified with these cell-based bioassays. In addition, the relative inducibility toward these drug metabolizing enzymes between therapeutic drugs, dietary chemicals and environmental pollutants was evaluated.

Materials and Methods. Human liver cDNA library was from Clontech (Palo Alto, CA). PCR-II vector for TA cloning, lipofectamin 2000, neomycin (G418), hygromycin, and culture media were purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Rifampicin, dexamethasone, mifepristone (RU-486), 2-AAF, and flavonoids (chrysin, apigenin, resveratrol and curcumin) were from Sigma (St. Louis, MO). Methoxychlor and mevastatin were from Biomol (Plymouth Meeting, PA). Other chemicals were obtained from Ultra Scientific (RI). Taq polymerase for PCR amplification, the dual luciferase detection kit, and the pRL-TK plasmid for transient transfection efficiency were purchased from Promega (Madison, WI). HepG2 and Caco-2 cell lines were from the American

Type Culture Collection (Manassas, VA). Primary human hepatocytes were a kind gift from Dr. Stephen Strom (Pittsburgh, PA). Unless otherwise noted, all DNA sequences are given in the 5' to 3' direction.

Northern blot analysis. Total RNA from primary human hepatocytes and from a PXR stable cell line was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and quantified by spectrophotometric analysis at 260 nanometers. Northern blots were performed by electrophoresis of 15 milligrams of total RNA through a 1 % agarose gel containing 2.2 moles per liter formaldehyde, followed by blotting onto nylon membranes (Molecular Simulation, Westboro, MA) (Shih *et al.* (1999) *Hum. Exper. Toxicol.*, 18:95-105). The RNA was crosslinked to the membranes using a Stratalinker ultraviolet crosslinker (Stratagene, La Jolla, CA) and the membranes hybridized to random-primed cDNA probes encoding human CYP1A1 or CYP3A4, as described in Allen *et al.* (2001) *Drug Metab. Disp.*, 29:1074-1079, and in Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 303:412-423 (both of which are herein incorporated by reference). Hybridization of blots was performed as described by Quattrochi *et al.* (1985) *DNA*, 4:395-400 (which is herein incorporated by reference) and then visualized by autoradiogram. The blot was subsequently washed and re-hybridized to an 18S rRNA probe to verify that equal amounts of total RNA had been applied to each lane.

Construction of dioxin response element in luciferase-SV40 vector. The core sequence of the AhR binding motif, the dioxin response element (DRE) was identified to be (T/G)NGCGTGA(A/C)(G/C)AA (**SEQ ID NO. 18**) (Lusska *et al.* (1993) *J. Biol. Chem.*, 268:6575-6580). Dioxin response elements have also been identified upstream of the regulatory region of other TCDD-inducible genes, including CYP1A2, CYP1B1, UDP-glucuronosyltransferase 1A1 (Yueh *et al.* (2003) *J. Biol. Chem.*, 278:15001-15006), and glutathione S-transferase Ya C (Whitlock (1999) *Annu. Rev. Pharmacol. Toxicol.*, 39:103-125). Two oligonucleotides, each containing three dioxin response elements were synthesized by Genset (San Diego, CA). The first oligonucleotide (**SEQ ID NO. 19**) had the sense sequence TTGCGTGCGATTGCGTGCGATTGCGTGCGA, which contains three repeats of the dioxin response element (DRE), TTGCGTGCGA (**SEQ ID NO. 20**). The second oligonucleotide (**SEQ ID NO. 21**) had the antisense sequence

TCGATCGCACGCAATCGCACGCAATCGCACGCAAGTAC, which contains three repeats of the dioxin response element (DRE), TCGCACGCAA (**SEQ ID NO. 22**). The underlined portion of each sequence represents the dioxin response element repeats. The second oligonucleotide (**SEQ ID NO. 21**) also incorporates linkers for the restriction enzyme sites KpnI and XhoI (shown as the non-underlined portions of the sequence). The synthesized oligonucleotides were phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), and the two phophorylated oligonucleotides were annealed together and directionally ligated into KpnI- and XhoI-digested luciferase gene containing a viral SV40 promoter and a neomycin resistance gene. Insertion of the dioxin response elements was verified by sequencing (Scripps Research Institute, La Jolla, CA). The luciferase-SV40 promoter vector, with the three copies of the dioxin response elements, was transfected into HepG2 cells, which were put under neomycin selection to yield the stable cell line DRE12-6, for detection of AhR-dependent transactivation.

Establishment of a stable cell line for CYP3A4 induction reporter assay.

1. *Generation of a stable cell line that overexpresses PXR.* Full-length PXR cDNA was cloned into the PCR-II vector (Invitrogen, Carlsbad, CA) by PCR, excised from the PCR-II vector by EcoRI restriction enzyme digestion, and subcloned into the expression vector pRIEShyg. The resulting plasmid was stably transfected into both human hepatoma HepG2 cells and colon carcinoma Caco-2, and hygromycin selection used to generate the stable transformants, Hep-PXR and Caco-PXR, respectively.

2. *Construction of CYP3A4 promoter reporter plasmid:* A 5'-flanking region of CYP3A4, known to contain the PXRE (-595 to +137 base pairs, with the transcription start site designated position 1), was amplified by PCR, using the sense primer: AAATAAGCTTGAGGAGCTCACCTCTG (**SEQ ID NO. 23**) and the antisense primer: AGGTTTCCATGGCCAAGTCTGGGAT (**SEQ ID NO. 24**). In addition, a distal enhancer module, XREM, was amplified by PCR using the sense primer ATTTGAGCTCTGGGGTCCCCCTTG (**SEQ ID NO. 25**), complementary to bases –7894 to –7871, and the antisense primer CACAGCTAGCAATGATCAAAGATGAC (**SEQ ID NO. 26**), complementary to bases –7106 to –7131; the primer sequences were based on the XREM

sequence deposited with GenBank with accession number AF185589. The resulting amplified XREM fragment was ligated upstream of PXRE containing a 3A4 promoter, into a luciferase vector.

A neomycin resistance gene was generated by PCR amplification using the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA), which encodes a neomycin resistant gene, as a DNA template. The sense primer had the sequence TAAAGTCGACAAAAATTTAACGCG (**SEQ ID NO. 27**), and the antisense primer had the sequence AGAGGTCGACGGTATACAGAC (**SEQ ID NO. 28**). Amplification was followed by SalI digestion of the PCR product, and the resulting fragment ligated to the SalI-digested PGL3-promoter plasmid, which contains luciferase as a reporter gene. The responsiveness of PXR and CYP3A4 response elements in response to PXR ligands was examined by transient transfection experiments with rifampicin and measured by dual luciferase detection kit. The CYP3A4 cell line DPX2 (Hepg-hPXR/3A4-Luc) was established by double transfection with the PXR expression plasmid and the luciferase plasmid containing CYP3A4 PXR response element under selection with both neomycin and hygromycin. *Luciferase assay:* Luciferase assays using the LucLite system (Packard Instrument Co., Meriden, CT) were performed according to the manufacturer's instructions. Results were expressed as relative light units or fold induction, relative to DMSO-treated control cells. The data represent the mean ± standard deviation of experiments performed in duplicate.

Results: To study AhR-mediated CYP1A1 induction, primary hepatocytes were treated with environmental polycyclic hydrocarbons and derivatives or with phytochemicals for 24 hours, followed by RNA isolation and Northern blot analysis (**FIG. 23A** and **23B**). The maximum induction of CYP1A1 was obtained with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at 10 nanomoles per liter, 3-methylcholanthrene (3-MC) at 0.5 nanomoles per liter, and the therapeutic agent omeprazole at 100 micromoles per liter, which were utilized as positive controls. With DMSO treatment as a control, no CYP1A1 induction is detected. By comparison, the phytochemically induced increase in CYP1A1 levels as seen by Northern blot analysis was small for chrysin (25 micromoles per liter) and curcumin (50 micromoles per liter) and negligible for resveratro (25 micromoles per liter). Similarly, treatments of the PAH derivative,

dimethylbenzo(a)pyrene (DMBA) (20 micromoles per liter), and the environmental arylamine, 2-acetylaminofluorene (2-AAF) (100 micromoles per liter), increased CYP1A1 expression slightly.

Time-course and dose-response relationship for DRE12-6 luciferase assay: A cell-based bioassay was developed to assess the arylhydrocarbon receptor (AhR) ligand accurately and efficiently. The stable cell line, DRE12-6, carrying three copies of the dioxin response elements, was grown in 96-well plates and treated with 10 nanomoles per liter TCDD at 4, 20, 24, and 28 hour time points. The maximum induction was obtained at the 28 hour time point where TCDD treatment resulted in a 20-fold induction of luciferase activity (**FIG. 24A**). In addition, DRE12-6 cells were treated with various concentrations of TCDD and 3-MC for 28 hours, which produced a dose-dependent increase in luciferase expression (**FIG. 24B**).

Effect of environmental chemicals and herbals on AHR-dependent and TCDD-mediated transcriptional activation: DRE12-6 stable transformants were utilized to examine AhR-mediated induction of luciferase expression by natural products, including flavonoids and over-the-counter health supplements (**FIG. 25A**), and by environmental contaminants, including PAHs, PCBs, DDT derivatives, and the arylamine 2-AAF (**FIG. 26A**). The relative potency and efficacy of these chemicals for eliciting an AhR-mediated response is presented relative to a control (DMSO treatment). Of the natural products examined, the flavonoid, chrysin, acted as a weak inducer compared with the positive control, TCDD. Omeprazole (25 micromoles per liter), was the most potent inducer (3.5 ± 0.051-fold above DMSO-treated cells).

The ability of these chemicals to affect TCDD-mediated transcriptional activation was further examined. DRE12-6 cells were treated with the test compounds for 3 hours prior to treatment for 28 hours with TCDD. This was followed by cell lysis and luciferase measurement. With TCDD co-treatment, kava-kava acted as a potent antagonist, decreasing TCDD-mediated induction from 12.99 ± 0.64 to 1.51 ± 0.06-fold. All tested flavonoids (quercetin, curcumin, chrysin, resveratrol, kaempferol, and apegenin) and green tea extract (GTE) also effectively inhibited TCDD-mediated induction as shown in **FIG. 25B**.

The luciferase activity in response to GTE obtained by the reporter assay in stable transformants was compared to expression studies with primary hepatocytes, a commonly used system for studying cytochrome P450 expression. Northern blot analysis with GTE-treated

human primary hepatocytes showed that treatment with GTE alone did not activate CYP1A1 expression, and co-treatment with TCDD and GTE resulted in the inhibition of TCDD-mediated induction, which agreed with the luciferase reporter assay (**FIG. 27**). Of the environmental contaminants tested, benzanthracene was a strong inducer, producing a 28.69 ± 1.65-fold induction in luciferase expression. In addition, 2-AAF (100 micromoles per liter) and DMBA (20 micromoles per liter) induced luciferase expression to 10.21 ± 0.36 and 2.23 ± 0.07-fold, respectively. Other test compounds were generally ineffective at activating AhR. However, in combination with TCDD, PCBs, such as arochlor 1254, 2,3,4,5 tetrachlorobiphenyl, and the DDT derivative methoxychlor, effectively inhibited TCDD-mediated induction.

PXR-medicated activation of CYP3A4: Regulation of CYP3A4 expression in response to various compounds was first studied in primary hepatocytes. Human primary hepatocytes were treated with herbal products for 48 hours, followed by RNA isolation and Northern blot analysis (**FIG. 28A** and **FIG. 28B**). The known human PXR activators, rifampicin (10 micromoles per liter), omeprazole (100 micromoles per liter), and phenobarbital (1000 micromoles per liter), increased CYP3A4 gene expression as shown in Northern blot analysis. Treatment with other phytochemicals resulted in only very slight or undetectable increase in CYP3A4 mRNA levels (**FIG. 28A**) suggesting that Northern blot analysis is a qualitative rather than a quantitative tool. In a separate Northern blot experiment, of the over-the-counter supplements tested, kava-kava produced a dramatic increase in CYP3A4 mRNA levels (**FIG. 28B**). Levels of CYP3A4 mRNA produced by treatment with other natural products were similar to that seen with DMSO treatment.

The responsiveness of CYP3A4 stable cells: Both the PXR expression vector and the luciferase reporter vector carrying CYP3A4 response elements were stably transfected into HepG2 and Caco-2 cells to generate the stable cell lines DPX2 (Hepg-hPXR/3A4-Luc) and Caco-3A4Luc, respectively. The cell-based bioassays were established in a 96-well format based on PXR-dependent activation for luciferase expression. Prior to establishing the stable cell lines, transient transfection experiments confirmed that stable transformants produced a dose-dependent induction by rifampicin of luciferase expression. In addition, responsiveness of DPX2 stable cells that overexpress PXR was examined by Northern Blot Analysis. Rifampicin induces

CYP3A4 in a concentration-dependent manner, indicating that CYP3A4 induction is both ligand- and PXR-dependent. In addition, CYP3A4 was efficiently activated by PXR ligands such as mevastatin and mifepristone, but not by pregnenolone-16-carbonitrile (PCN), a strong activator of rodent PXR (**FIG. 29**).

The cell-based bioassays were developed in a high-throughput system using stable transformants grown in 96- or 384-well microtiter plates. Various compounds were tested for their ability to induce luciferase activity, including known PXR ligands and conventional drugs (**FIG. 30A**), environmental contaminants (**FIG. 30B**), and herbal supplements (**FIG. 30C**). Known PXR ligands, including rifampicin, dexamethasone, and clotrimazole, produced dose-dependent luciferase response curves in DPX2 cells (**FIG. 30A**) as did the environmental contaminant methoxychlor (**FIG. 30B**) and the herbal supplement kava-kava (**FIG. 30C**). Interestingly, kava-kava (100 micrograms per milliliter) was more potent than rifampicin, inducing luciferase activity 33.2-fold above DMSO treatment.

Finally, herbal products and environmental contaminants were examined for their inducibility on CYP3A4 expression in DPX2 cells. Of the compounds tested, the flavonoid chrysin (25 micromoles per liter), activated PXR receptor and luciferase activity by 9.8-fold (**FIG. 30C**). Similarly, 2-AAF, an environmental contaminant, induced luciferase expression by 24.8-fold (**FIG. 30B**).

The cell-based bioassays described above are capable of identifying herbal supplements and environmental chemicals that induce receptor-mediated genes, including CYP1A1, CYP1A2, and CYP3A4, and are suitable to use in a high throughput system. Of the chemicals tested, the plant flavonoid chrysin was found to activate both AhR and PXR receptors, and enhanced luciferase activity mediated by the CYP1A and CYP3A4 response elements by 4.2- and 9.8-fold, respectively. Similarly, the environmental contaminant, 2-acetylaminofluorene induced CYP1A and CYP3A4 genes by 4.8- and 24.7-fold, respectively. Xenochemical induction of drug metabolizing enzymes, as measured by the reporter assay in stable transformants, was confirmed by a conventional mRNA induction assay using primary hepatocytes, indicating that results were comparable between these two assays. Thus, the cell-based bioassays described above can

replace conventional detection of mRNA levels as a convenient and reliable bioassay for predicting potential xenobiotic-mediated interactions with traditional drugs.

EXAMPLE VI

Transcriptional Activation of *MDR1* (P-glycoprotein) by PXR Ligands

The human *MDR1* gene encodes a 170 kilodalton phosphorylated and glycosylated membrane protein, P-glycoprotein (P-gp). Pharmaceutical agents can be inactivated by hepatic drug-metabolizing enzymes, and also excreted from the intestine by transporter proteins, such as P-glycoprotein, which can actively expel a large variety of drugs from cells, leading to a decreased intracellular drug concentration (Higgins (1992) *Annu. Rev. Cell. Biol.,* 8:67-113; Sharom (1997) *Biochem. Soc. Trans.,* 25:1088-1096). P-glycoprotein is expressed in tissues involved in absorption, excretion, and distribution (Thiebaut *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.,* 84:7735-7738). The effect of P-glycoprotein on drug efficacy has been demonstrated in *mdr1a* -/- mice, where systemic bioavailability of orally administered drugs was markedly increased in comparison to wild-type mice (Sparreboom *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.,* 94:2031-2035; Mayer *et al.* (1996) *Br. J. Pharmacol.,* 119:1038-1044).

Although PXR-mediated P-glycoprotein activation can limit exposure to environmental or dietary toxins, P-glycoprotein may also hinder the uptake of therapeutic drugs, increasing the possibility of multidrug resistance and possibly causing the failure of chemotherapy trials (Gottesman *et al.* (1994) *Ann. N.Y. Acad. Sci.,* 716:126-138; Ambudkar *et al.* (1999) *Annu. Rev. Pharmacol. Toxicol.,* 39:361-398). Drug-drug interactions can arise due to concurrent administration of drugs which are both inducer and substrate for CYP3A4 and MDR1 P-glycoprotein. For example, St. John's Wort, a over-the-counter supplement that activates PXR, when co-administered with conventional drugs, elevated expression of P-glycoprotein in the intestine and expression of CYP3A4 in the intestine and liver, lowering the blood concentration and decreasing the efficacy of the drugs (Dürr *et al.* (2000) *Clin. Pharmacol. Ther.,* 68:598-604).

The nuclear receptor PXR (pregnane X receptor; NR1I2) binds to a PXR response element in the regulatory region of the CYP3A4 gene in response to a diverse array of chemicals

(Bertilsson *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.*, 95:12208-12213; Kliewer *et al.* (1998) *Cell*, 92:73-82; Lehmann *et al.* (1998) *J. Clin. Invest.*, 102:1016-1023; Lewis *et al.* (2000) *J. Steroid Biochem. Mol. Biol.*, 74:179-185), inducing CYP3A4 expression and altering the metabolic profile for many compounds. CYP3A4 and MDR1 P-glycoprotein are co-induced by the same set of chemicals (Synold *et al.* (2001) *Nature Med.*, 7:584-590); PXR also plays a central role in regulating P-glycoprotein transcription (Geick *et al.* (2001) *J. Biol. Chem.*, 276:14581-14587; Synold *et al.* (2001) *Nature Med.*, 7:584-590). By DNA binding assays and transfection experiments, several PXR binding sites were identified that form a complex cluster located about 8 kilobases upstream of the *MDR1* regulatory region. A direct repeat (DR4) nuclear receptor response element was identified within this cluster, and found to be essential for *MDR1*induction by rifampicin (Geick *et al.* (2001) *J. Biol. Chem.*, 276:14581-14587).

The present invention shows that nuclear receptor PXR-mediated induction of MDR1 P-glycoprotein is cell type-specific. The activation profiles of *MDR1*and *CYP3A4*, major target genes of PXR, were compared. The present invention describes a stable cell line-based assay, suitable to high-throughput screening to identify ligands that interact with PXR and induce MDR1 transcriptional activity.

Materials and Methods. Human liver cDNA library was from Clontech (Palo Alto, CA). PCR-II vector for TA cloning, lipofectamin 2000, neomycin (G418), and culture media were purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Rifampicin, dexamethasone, mifepristone (RU-486), 2-AAF, and flavonoids (chrysin, apigenin, resveratrol and curcumin) were from Sigma (St. Louis, MO). Methoxychlor and mevastatin were from Biomol (Plymouth Meeting, PA). Taq polymerase for PCR amplification, the dual luciferase detection kit, and the pRL-TK plasmid for transient transfection efficiency were purchased from Promega (Madison, WI). HepG2, Caco-2, and LS174-T cell lines were from the American Type Culture Collection (Manassas, VA). Primary human hepatocytes were a kind gift from Dr. Stephen Strom (Pittsburgh, PA). Unless otherwise noted, all DNA sequences are given in the 5' to 3' direction.

Cloning of human PXR cDNA and DR4 motif in regulatory region of MDR1 gene (P-glycoprotein): Human PXR cDNA was cloned from a human liver cDNA library (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482) and inserted into a pIRESneo expression vector. The promoter region of the *MDR1* gene, which contains a DR4 motif, was amplified using human genomic DNA as a template. Primers were designed based upon the human *MDR1* sequence deposited with GenBank with the accession number AC002457. The sense primer had the sequence ATAAGGTACCAACTGTTCATTGGTC (**SEQ ID NO. 29**), and the antisense primer had the sequence AATTCTCGAGCTTATAAAAACACCA (**SEQ ID NO. 30**). Restriction enzyme sites KpnI and XhoI were incorporated at the 5' ends of the sense and antisense primers, respectively. PCR was performed with the following conditions: initial denaturation at 94 degrees Celsius (2 minutes), followed by 30 denaturation/annealing/extension cycles of 94 degrees Celsius (20 seconds), 58 degrees Celsius (30 seconds), and 72 degrees Celsius (90 seconds). This was followed by a final extension of 7 minutes at 72 degrees Celsius. A 239 base pair DNA fragment, from –7990 to – 7752 bases from the transcription initiation site of MDR1, was generated and cloned into KpnI/XhoI double-digested luciferase reporter vector.

Northern Blot analysis for MDR1 P-glycoprotein and CYP3A4 expression: Total RNA was prepared from cells (primary hepatocytes, LS174T, HepG2, or Caco-2) using Trizol reagent (Invitrogen, Carlsbad, CA). For Northern blots, 15 micrograms of total RNA was fractionated on a 1% formaldehyde gel and transferred onto a nylon membrane by capillary transfer. A 530-base pair fragment of *MDR1* cDNA was obtained from PCR cloning using the sense primer GGTTGGAAGCTAACCCTTGTGATTT (**SEQ ID NO. 31**) and the reverse primer CGAGATGGGTAACTGAAGTGAACAT (**SEQ ID NO. 32**), and a human liver cDNA library as the template. The resulting fragment was ^{32}P-labeled by random priming (Invitrogen, Carlsbad, CA) for use as an *MDR1* probe. A CYP3A4 cDNA probe was made similarly (Raucy *et al.* (2002) *J. Pharmacol. Exp. Ther.*, 302:475-482). All constructs and cDNAs were subjected to sequence analysis. Hybridization was performed with the ExpressHyb system according to the manufacturer's instructions (Clontech, Palo Alto, CA).

PXR quantification by RT-PCR: PXR mRNA levels were quantified by reverse transcription coupled to amplification of cDNA by the polymerase chain reaction (RT-PCR). Total RNA was

isolated from various cell lines, including primary human hepatocytes, LS174T, HepG2, HepG2 overexpressing PXR (Hep-PXR), Caco-2 and Caco-2 overexpressing PXR (Caco-PXR), using trizole (Invitrogen, Carlsbad, CA), and quantified based on absorbance at 260 nanometers. Conditions for the RT-PCR reaction were based on the Access RT-PCR Kit (Promega, Madison, WI) manufacturer's recommendations. Briefly, an aliquot of total RNA was used as a template for first-strand synthesis using a gene-specific reverse primer, TCCCGAAAGATCTGTGCTCTT (**SEQ ID NO. 33**), and Avian Myeloblastosis Virus (AMV) reverse transcripatase. The reverse transcriptase reaction was followed by PCR using the same reverse primer (**SEQ ID NO. 33**) and the forward primer, AGTCTCTTCCAAGCAGTAGGA (**SEQ ID NO. 34**), with *Tfl* DNA polymerase. These forward and reverse primers are complementary to sequences found in Exon 5 and Exon 6 of the PXR gene, respectively, to preclude the possibility of genomic DNA contamination. The template RNA was serially diluted in water from 20 nanograms to 10 picograms per reaction. The expected partial cDNA (312 base pairs) product was visualized by agarose gel electrophoresis with ethidium bromide staining.

Western Blot analysis: Cell lysates were isolated from the various cell lines by the method of Guengerich ("Analysis and characterization of enzymes" in "Principles and Methods of Toxicology", A. W. Hayes, editor, Raven Press, New York, 1989, pp. 777-814) and protein concentrations determined by with a BCA kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Cell lysates from human cell lines were resolved by SDS-PAGE (Laemmli, 1970) and then electrophoretically transferred to nitrocellulose (Towbin *et al.*, 1979). MDR1 P-glycoprotein was detected utilizing a rabbit polyclonal antibody (Research Diagnostics, NJ) to human P-glycoprotein as the primary antibody and horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) as the secondary antibody with visualization using a chemiluminescence kit (Pierce Chemical Co., Rockford, IL).

Generation of stable cell lines that overexpress PXR: Full length PXR cDNA was cloned into a PCR-II vector (Invitrogen, Carlsbad, CA) by PCR, excised from the PCR-II vector by EcoRI restriction enzyme digestion, and subcloned into the expression vector pRIESneo, which was stably transfected into both hepatoma HepG2 cells and colon carcinoma Caco-2 to generate the stable transformants Hep-PXR and Caco-PXR cells, respectively.

Transient transfection assay and reporter gene assay: The 239 base pair DNA fragment from the 5' regulatory region of *MDR1* and containing the DR4 motif was cloned by PCR. This was ligated upstream of a luciferase reporter gene in a plasmid that contained the heterologous SV40 promoter to drive transcription of pgp-DR4, producing the mdr1(dr4)-Luc construct. Transient transfection experiments were carried out using both recombinant luciferase plasmid and the PXR expression vector, followed by a 48 hour treatment with various compounds. Cells were co-transfected with *Renilla* luciferase to normalize transfection efficiency. HepG2 or Caco-2 cells were plated in 24-well tissue culture plates at a density of 5 x 10^4 cells per well and transfected after 24 hours using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. In general, transfection mixtures contained 200 nanograms of mdr1(dr4)-Luc plasmid, 200 nanograms of PXR expression plasmid, and 100 nanograms of *Renilla* luciferase plasmid (as an internal control to monitor for transfection efficiency). The day after transfection, the cells were treated with chemicals for 48 hours, then harvested, lysed, and analyzed for luciferase activity, which was assessed by a dual luciferase kit (Promega, Madison, WI) using the Lumistar galaxy luminometer (BMG Labtechnologies). Reporter activity was normalized to the *Renilla* luciferase control.

Establishment of stable transformants containing both PXR and reporter plasmid and development of high throughput bioassay for PXR-dependent transactivation: The stable transformants Hep-PXR/mdr1(dr4)-Luc and Caco-PXR/mdr1(dr4)-Luc cell lines were generated from the parental cell lines HepG-PXR and Caco-PXR, respectively, according to the following procedure. The parental cell lines HepG-PXR and Caco-PXR, which overexpress PXR, were stably transfected with the luciferase reporter plasmid mdr1(dr4)-Luc. The transfected cells were cultured in DMEM (HepG-PXR cells) or in IMEM (Caco-PXR cells), both media supplemented with 10% fetal bovine serum and neomycin (700 micrograms per milliliter). Positive foci (resistant to neomycin) were picked, expanded, and assayed for their ability to respond to rifampicin over DMSO treatments.

For the induction assay, stable transformants were seeded in 96-wells plates at a density of 1 x 10^4 cells per well and allowed to recover overnight. Plates were treated with test compounds at varying concentrations, using 3 replicates per dose. After 48 hours of exposure to

the test compound, cells were washed with phosphate buffered saline, lysis buffer was applied to the wells, and luciferase substrate added. Following 10 minutes for light adaptation, luminescence was monitored according to the manufacturer's instructions (Packard kit, Biomed). Luciferase activity induced by selective chemical treatment, normalized to that seen with a DMSO treatment control, indicated the relative level of *MDR1* induction.

Results:

MDR1 P-glycoprotein expression profile in response to PXR activators in LS174T, Caco-2, HepG2 cells and primary human hepatocytes: To demonstrate that nuclear receptor PXR regulation is cell type-specific, its ability to activate MDR1 P-glycoprotein expression was compared with a series of PXR agonists in various liver and gut cell lines and primary human hepatocytes by Northern and Western blot analyses. In colon adenocarcinoma LS174T cells, *MDR1* is expressed at low constitutive levels and is effectively induced by PXR activators, including rifampicin, omeprazole, RU-486 (mifepristone) and dexamethasone (**FIG. 31**). Treatment with pregnenolone 16 alpha-carbonitrile (PCN), a specific agonist of the mouse ortholog of PXR, resulted in only a slightly increase in mRNA levels (**FIG. 31**, lane 7), consistent with the observation that PCN acts as a very weak PXR-mediated inducer of human CYP3A4 (Kocarek *et al.* (1995) *Drug Metab. Disp.*, 23:415-421; Quattrochi *et al.* (1995) *J. Biol. Chem.*, 270:28917-28923). MDR1 P-glycoprotein was effectively activated in primary human hepatocytes by rifampicin treatment, a result similar to that seen in LS174T cells (**FIG. 32A**).

Activation of MDR1 P-glycoprotein expression via chemical treatment is presumably mediated by the nuclear receptor PXR as described previously (Geick *et al.* (2001) *J. Biol. Chem.*, 276:14581-14587; Huang *et al.* (2001) *Drug Metab. Disp.*, 29:754-760; Schuetz *et al.* (1995) *Mol. Carcinogen.* 12:61-65; Schuetz *et al.* (1995) *J. Cell Physiol.*, 165:261-272). Hepatoma HepG2 and colon Caco-2 cells have been shown to respond poorly to PXR activators where there are low levels or a lack of endogenous PXR (Xie *et al.* (2003) *Proc. Natl. Acad. Sci. U.S.A.*, 100:4150-4155). In order to study PXR-dependent regulation of MDR1 expression, stable transformants, Hep-PXR and Caco-PXR, were prepared to constitutively express human

PXR in HepG2 and Caco-2 cells, respectively. As expected, treatment of the HepG2 or Caco-2cells with rifampicin caused no changes on the expression of MDR1 P-glycoprotein, due to low or lack of endogenous PXR (**FIG. 32B**). Interestingly, high constitutive levels of MDR1 P-glycoprotein were detected both in DMSO- and in rifampicin-treated HepG2 cells. Similarly, in Hep-PXR stable cells, MDR1 P-glycoprotein exhibited high constitutive levels with DMSO treatment, and treatment with rifampicin did not induce MDR1 P-glycoprotein expression further (**FIG. 32B**). In contrast, MDR1 P-glycoprotein activation was followed by rifampicin treatment in Caco-PXR stable cells, but not in parental Caco-2 cells, indicating the essential role of PXR (**FIG. 32C**).

Expression of CYP3A4, another target gene of PXR, was compared to that of MDR1 P-glycoprotein (**FIG. 32A**, **32B**, and **32C**, middle panel). CYP3A4 was activated effectively in primary hepatocytes with rifampicin. Treatment of the HepG2 or Caco-2 cells with rifampicin caused very slight or no changes to the expression of CYP3A4, consistent with the fact that the activation is PXR-dependent. Stably transfected Hep-PXR cells exhibit the same activation profiles as primary hepatocytes, where CYP3A4 expression was profoundly induced in response to the PXR ligand, rifampicin, demonstrating that PXR acts as a key regulator of CYP3A4 expression in the target organ, liver. In contrast, CYP3A4 responded poorly to rifampicin in both colon cell lines LS174-T and Caco-PXR (the latter overexpressing PXR), indicating that liver cells may be a more sensitive model for CYP3A4 activation than are colon cells.

The sensitivity of PXR-mediated activation of MDR1 P-glycoprotein expression to endogenous PXR level was investigated. RT-PCR was performed and mRNA concentration-dependent reverse transcription and cDNA synthesis was visualized on agarose gel (**FIG. 33A**). The amounts of total RNA template used in the RT-PCR reaction were lowest for primary hepatocytes (10, 40, and 200 picograms); 0.02 to 2 nanograms total RNA were used for other cell types. Primary human hepatocytes expressed the highest level of PXR (relative to the amount of RNA template used) among the cell lines examined. The second highest PXR expression level was observed for Hep-PXR, HepG2, and LS174T cells, where a 100-fold greater amount of RNA template was used to obtain visible cDNA products; PXR mRNA was undetectable in Caco-2 cells under these conditions. It is possible that nuclear PXR might be more abundant in LS174T

cells; this may be investigated by performing Western blot analyses of nuclear and cytosolic fractions. Finally, the response of endogenous PXR levels to PXR ligands were examined in LS174T cells by RT-PCR. Total PXR levels were unchanged between test compound- and DMSO-treated LS174 cells, suggesting that PXR ligands activate PXR in LS174 cells by translocation rather than by increasing PXR expression levels (**FIG. 33B**). The different expression profiles between MDR1 P-glycoprotein and CYP3A4 in response to PXR ligands in LS174-T cells suggest that, in addition to PXR, other cell type-specific proteins coordinate with PXR to regulate MDR1 P-glycoprotein expression in LS174T cells. Primary hepatocytes are physiologically and biochemically most similar to intact liver cells and remain the most promising approach currently available for predicting induction of CYP3A4 and MDR1 P-glycoprotein in response to foreign compounds. Colon cell lines (such as LS174T and Caco-PXR) may be more sensitive cellular models for monitoring PXR-mediated induction of MDR1 P-glycoprotein.

Up-regulation of MDR1 P-glycoprotein transcriptional activity by cotransfection of mdr1(dr4)-luciferase plasmid and PXR expression vector in transient transfection experiments: To identify and verify a PXR response element in the promoter region of the *MDR1* gene, a DNA cluster containing a DR4 motif was cloned by PCR from human genomic DNA and ligated into the multiple cloning site of a plasmid containing a luciferase gene. This PXR response element-containing recombinant plasmid was co-transfected transiently with either of the nuclear receptors PXR or CAR (constitutive androstane receptor) into Caco-2 or HepG2 cells. Expression of luciferase activity was determined after treatment of cells for 48 hours (sufficient time for adequate accumulation of both RNA and protein in PXR ligand-treated cells). A 3- to 6-fold increase in luciferase activity was observed in Caco-2 cells after treatment with various PXR activators (rifampicin, mifepristone, omeprazol, and mevastatin) (**FIG. 34A**), indicating that the mdr1(dr4) sequence was transactivated by PXR. Treatment with PCN, a potent activator of rodent PXR, resulted in only a minor increase in luciferase activity in Caco-2 cells (**FIG. 34A**). In contrast, transient co-transfection with CAR did not produce induction of luciferase expression with the same set of chemical treatments. With rifampicin treatment, HepG2 cells overexpressing PXR produced a dose-dependent induction in luciferase expression (**FIG. 34B**).

Reporter assay for measurement of PXR-dependent transactivation in stable cells: MDR1 P-glycoprotein induction is associated with a decrease in anti-cancer drug efficacy and increased resistance to drugs used in cancer treatments. Assays that detect MDR1 P-glycoprotein induction and PXR activation have important implications for drug design with regard to drug interaction. Primary human hepatocytes could serve as a cellular model for studying MDR1 P-glycoprotein induction, but inter-individual variability in primary cell cultures and the difficulty in obtaining cells are drawbacks. Also, traditional mRNA detection methods are labor-intensive and more suited to qualification than quantification. Transient transfection methods, such as described above, involve co-transfection with internal control plasmids, a process that is tedious and may yield results variable between experiments.

A simple and reliable assay was developed based on the ability of test compounds to induce PXR-mediated transcription of MDR1 P-glycoprotein in stable cell lines. Incorporation of both a PXR expression vector and a luciferase reporter plasmid containing the PXR response element mdr1(dr4), into either HepG2 or Caco-2 cells, generated the stable cell lines Hep-PXR/Pgp-Luc or Caco-PXR/Pgp-Luc, respectively.

The cell-based bioassays were developed in a high-throughput system using stable transformants (Hep-PXR/Pgp-Luc or Caco-PXR/Pgp-Luc) grown in a 96- or 384-well format. Treatment with the well known PXR ligands rifampicin, dexamethasone, mevastatin, mifepristone (RU-486), androstanole, and omeprazole, produced dose-dependent luciferase induction in both Hep-PXR/Pgp-Luc (**FIG. 35A**) and Caco-PXR/Pgp-Luc (**FIG. 35B**). Omeprazole, tested at 100 micromoles per liter, was observed to be as potent in inducing luciferase activity in Hep-PXR/PgpLuc cells as was rifampicin at 10 micromoles per liter (**FIG. 35A**). Weaker luciferase induction was seen with dexamethasone in Hep-PXR/PgpLuc cells (**FIG. 35A**). Caco-Pxr/PgpLuc stable cells appeared to be generally more responsive toward PXR activators than were Hep-PXR/PgpLuc stable cells, suggesting that cell type- and/or tissue-specific factors might be involved. Mevestatin and mifepristone were observed to strongly induce luciferase activity in Caco-PXR/Pgp-Luc cells. These results of a stable cell line-based reporter assay using Hep-PXR/PgpLuc and Caco-PXR/Pgp-Luc cells concur with results obtained by Northern Blot analysis of LS174T cells.

Finally, the stable transformant Hep-PXR/Pgp-Luc was used to study the ability of environmental contaminants and natural herbal products to induce MDR1 P-glycoprotein expression (**FIG. 36**). Of the natural products examined, the herbal supplement kava-kava and the flavonoid chrysin were observed to be the most potent inducers, increasing luciferase activity by 5 fold. Rifampicin (a known PXR activator), 2-acetylaminofluorene (2AAF, an environmental contaminant), and methoxychlor (a DDT derivative) also strongly induced luciferase activity (**FIG. 36**).

Finally, *MDR1* gene regulation in response to chrysin was studied in primary hepatocytes by Northern blot analysis. Human primary hepatocytes were exposed to 0.1% dimethylsulfoxide (DMSO) as a vehicle control, to the known PXR ligand rifampicin (Rif) (10 micromoles per liter), or to the flavonoid chrysin (25 micromoles per liter) for 48 hours. Chrysin was observed to induce the expression of both MDR1 and CYP3A4 (**FIG. 37**).

The above results demonstrate that nuclear receptor PXR-mediated induction of MDR1 P-glycoprotein is cell type-specific. MDR1 P-glycoprotein was more effectively induced by PXR ligands in primary human hepatocytes and LS174T colon adenocarcinoma cells, than in HepG2 and Caco-2 cells. The degree of MDR1 P-glycoprotein induction by a PXR activator was found to be not always proportional to endogenous PXR expression levels. In HepG2 hepatoma cells, high endogenous levels of MDR1 P-glycoprotein were observed, but rifampicin, a known PXR ligand, did not further induce MDR1 P-glycoprotein expression. Rifampicin treatment did not induce MDR1 P-glycoprotein expression in Caco-2 primary colonic tumor cells, in contrast to the induction seen in LS174T cells. When Caco-2 cells that overexpress PXR were incubated with a set of known PXR ligands, they showed a ligand concentration-dependent induction of luciferase reporter activity which indicates the involvement of PXR in this induction. CYP3A4, a gene also induced by PXR, exhibits an activation profile different from that seen for MDR1 P-glycoprotein, in response to rifampicin.

Transient transfection experiments were performed to verify that the transcriptional activation of *MDR1* is both PXR- and response element (DR4)-dependent. Stable cell lines Hep-PXR/PgpLuc and Caco-PXR/Pgp-Luc, incorporating both a PXR-expression vector and a luciferase reporter containing a PXR response element, were constructed in HepG2 and Caco-2

cells, respectively. These stable cell lines can be employed in cell-based bioassays, such as those described above, that may be useful in accurately and rapidly predicting MDR1 P-glycoprotein inducibility by test compounds, such as new chemical entities. Such cell-based bioassays are suitable to high-throughput screening of compounds to rapidly identify ligands that interact with PXR and induce MDR1 transcriptional activity. The cell-based bioassays described above were shown to be capable of identifying xenobiotics that activate PXR-mediated MDR1 P-glycoprotein induction. These results are in agreement with those obtained by the more tedious method of Northern blot analysis of mRNA induction.

All publications, including patent documents and scientific articles, referred to in this application, including any bibliography, are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication were individually incorporated by reference.

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